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(54) Title: ADENOVIRUS GENE EXPRESSION SYSTEM

(57) Abstract

The present invention provides a novel, highly efficient, recombinant adenovirus expression system for expression of a heterologous gene(s) and/or gene product(s) in a mammalian cell. The recombinant adenovirus was produced by cotransfecting a novel vector with the large fragment of the adenovirus-5 genome in 293 cells. Homologous recombination between these two DNA fragments resulted in the production of the recombinant adenovirus expression system. This vector, when converted to a recombinant virus has the unique capability of expressing one or more heterologous genes at very high levels. The novel vector, comprises, at least one cDNA insertion site for cloning a selected heterologous gene; a promoter sequence positioned upstream from the gene insertion site; the left end replication and packaging elements of the adenovirus-5 genome positioned upstream of the promoter; a highly efficient eukaryotic splice acceptor and splice donor site positioned immediately downstream of the promoter; and positioned downstream of the insertion site a strong polyadenylation sequence and the region for homologous recombination containing a portion of the adenovirus-5 genome. Between the packaging sequence and the CMV promoter are restriction sites for insertion of a second fully functional transcription unit.

ADENOVIRUS GENE EXPRESSION SYSTEM

FIELD OF THE INVENTION

The present invention relates generally to a recombinant viral expression system. More particularly, the present invention relates to a highly efficient, recombinant adenovirus expression system capable of expressing a heterologous gene(s) in a host mammalian cell.

BACKGROUND OF THE INVENTION

The human adenovirus-5 (Ad5) genome consists of a double-stranded linear DNA molecule of 36 kilo-basepair (bp) (Ginsberg, 1984). The virus replication cycle has two phases: an early phase, during which 4 transcription units E1, E2, E3, and E4 are expressed, and a late phase occurring after the onset of viral DNA synthesis when late transcripts are expressed from the major late promoter (MLP). These late messages encode most of the viral structural proteins. E1, E2 and E4 gene products of human adenoviruses are involved in transcriptional activation, cell transformation, and viral DNA replication as well as other viral functions, and are essential for viral growth (Grand, 1987, *Biochem. J.*, vol. 241, pp. 25-38; and Nevins, 1987, *Microbiol. Rev.*, vol. 51, pp. 419-430). In contrast, E3 gene products are not required for viral replication in cultured cells (Ginsberg et al., 1989,), but appear to be involved in evading immune surveillance in vivo (Anderson et al., 1985, *Cell*, vol. 43, pp. 215-222; Burgert et al., 1985, *Cell*, vol. 41, pp. 987-997; Burgert et al., 1987, *EMBO J.*, vol. 6, pp. 2019-2026; Carlin et al., 1989, *Cell*, vol. 57, pp. 135-144; Gooding and Wold, 1990, *Crit. Rev. Immunol.*, vol. 10, pp. 53-71; Gooding et al., 1988, *Cell*, vol. 53, pp. 341-346; Horton et al., 1990, *J. Virol.*, vol. 64, pp. 1250-1255; Tollefson et al., 1991, *J. Virol.*, vol. 65, pp. 3095-3105; Wold and Gooding, 1989, *Mol. Biol. Med.*, vol. 6, pp. 433-452; and Wold and Gooding, 1991, *Virology*, vol. 184, pp. 1-8).

E1 and E3 and a site upstream of E4 have been utilized as sites for insertion of foreign DNA sequences in the generation of recombinant adenoviruses (Berkner et al.,

Adenovirus expression vectors have been in use for the past decade (Thummel et al., 1981, *Cell*, vol. 23, pp. 825-836; Berkner et al., 1984, *Nucleic Acids Res.*, vol. 12, pp. 1925-1941; and for a review see Grunhaus et al., 1992, *Seminars in Virology* 3, pp. 237-252), and more recently exploited for the purpose of gene therapy (Herz et al., 1993, *Proc. Natl. Acad. Sci. U.S.A.*, vol. 90, pp. 2812-2816; Rosenfeld et al., 1991, *Science*, vol. 252, pp. 431-434; and Rosenfeld et al., 1992, *Cell*, vol. 68, pp. 143-155). Features of adenovirus based expression vectors which make them attractive to gene therapy applications include very efficient uptake into cells which contain the appropriate adenovirus receptor and uptake pathway, and the ability to carry up to 7.5 kb of foreign DNA. Adenovirus vectors allow a reporter gene to be under the control of tissue specific promoter elements (Friedman et al., 1986, *Mol. Cell. Biol.*, vol. 6, pp. 3791-3797; and Babiss et al., 1986, *Mol. Cell. Biol.*, vol. 6, pp. 3798-3806) as well as a variety of viral and mammalian constitutive promoter elements (Mittal et al., 1993, *Virus Research*, vol. 28, pp. 67-90).

One such example of an adenovirus-based vector system is described in Mittal et al., 1993, *Virus Research*, vol. 28, pp. 67-90. The authors here describe a helper-independent adenovirus type 5-luciferase recombinant containing the firefly luciferase gene flanked by simian virus 40 (SV40) regulatory sequences inserted into the early region 3 (E3) of the adenovirus-5 genome. A plasmid containing the luciferase gene and SV40 regulatory sequences in the E3 region was co-transfected with a plasmid containing the adenovirus-5 d1309 genome in circular form. Upon transfection of 293 cells, virus progeny produced by *in vivo* recombination between the two plasmids resulted in rescue of the adenovirus type 5-luciferase recombinant (i.e., E3 insert in Adenovirus-5 genome).

Gomez-Foix et al., 1992, *J. Biol. Chemistry*, vol. 267, no. 35, pp. 25129-25134, discloses adenovirus-mediated transfer of the muscle glycogen phosphorylase gene into hepatocytes in culture. The preparation of a recombinant adenovirus containing the cDNA encoding rabbit muscle glycogen phosphorylase is described whereby the cytomegalovirus (CMV) early gene promoter/enhancer, pUC 18 polylinker, fragment of the SV40 genome that includes the small T-antigen intron and the polyadenylation

Rosenfeld et al., 1991, *Science*, vol. 252, pp. 431-434, discloses adenovirus-mediated transfer of recombinant α1-antitrypsin gene to the lung epithelium cells of the cotton rat respiratory tract *in vivo*. The adenoviral vector contained an adenovirus major late promoter and a recombinant human α1-antitrypsin gene.

Quantin et al., 1992, *Proc. Natl. Acad. Sci. USA*, vol. 89, pp. 2581-2584, discloses a recombinant adenovirus containing the β-galactosidase reporter gene under the control of muscle-specific regulatory sequences. This recombinant virus directed expression of the β-galactosidase in myotubes *in vivo*.

Problems associated with adenovirus infection, particularly those associated with repression of host cell mRNA translation and shutdown of host normal mRNA production (Babich et al., 1983, *Mol. Cell. Biol.*, vol. 3, pp. 1212-1221; Beltz et al., 1979, *J. Mol. Biol.*, vol. 131, pp. 353-373; Schneider et al., 1987, *Annu. Rev. Biochem.*, vol. 56, pp. 317-332) have been addressed by using defective adenovirus vectors which are based on mutations in the dominant regulatory region, E1 (Harrison et al., 1977, *Virology*, vol. 77, pp. 319-329; Jones et al., 1979, *Cell*, vol. 17, pp. 583-689). In addition, conventional adenovirus vector systems typically require high cell exposure (e.g., MOI's in excess of 500 PFU/cell) for expression of the desired gene, which is detrimental to the cells because of cytopathic effects from exposure. Therefore, a need exists for an adenovirus-mediated expression vector which can infect cells at low doses, yet can exhibit maximum expression of a gene in the cell.

Moreover, although adenovirus-based vectors for gene expression have been successfully employed with a number of mammalian and viral genes (for review, see Mulligan, R.C., 1993, *Science*, vol. 260, pp. 926-932), they have not apparently been used to express any member of the guanine nucleotide-binding protein coupled receptors (GPCR) family, such as the pituitary thyrotropin-releasing hormone (TRH-R)(Straub et al., 1990, *Proc. Natl. Acad. Sci U.S.A.*, vol. 87, pp. 9514-9518; Yamada et al., 1992, *Biochem. Biophys. Res. Commun.*, vol. 184, pp. 367-372; Zhao et al., 1992, *Endocrinology*, vol. 130, pp. 3529-3536; de la Pena et al., 1992, *Biochem. J.*, vol. 284, pp. 891-899). Seven transmembrane-spanning GPCRs comprise a large family of cell surface regulatory proteins (Dohlman et al., 1991, *Annu. Rev. Biochem.*,

which have successfully taken up sufficient quantities of DNA (Kitsis et al., 1991, *Proc. Natl. Acad. Sci. U.S.A.*, vol. 88, pp. 4138-4142; Lin et al., 1990, *Circulation*, vol. 82, pp. 2217-2221; and Ascadi et al., 1991, *The New Biologist*, vol. 3, pp. 71-81). The problem associated with direct DNA injection is its relative inefficiency as only approximately 0.02% of the myocytes in the adult rat heart take up and express injected DNA (Kitsis et al., 1993, in *Methods in Molecular Genetics*, ed. Adolph, K. W., Academic Press, Inc., New York, Vol. 1, pp. 374-392).

A recent report demonstrated efficient gene transfer into adult rat cardiocytes *in vitro* (Kirshenbaum et al., 1993, *J. Clin. Invest.*, vol. 92, pp. 381-387). In addition, recent studies using adenovirus vectors introduced intravenously into both rats and mice, indicate that the virus will infect a wide variety of tissue types, including mouse skeletal and cardiac muscle (Quantin et al., 1992, *Proc. Natl. Acad. Sci. U.S.A.*, vol. 89, pp. 2581-2584; and Strattford-Perricaudet et al., 1992, *J. Clin. Invest.*, vol. 90, pp. 626-630). However, little quantitative data is available concerning expression of adenovirus-mediated gene transfer *in vivo*. Therefor, a need exists for an adenovirus-mediated gene transfer vector system which would function effectively with primary cultures of cardiac myocytes and one which would also have application *in vitro*.

SUMMARY OF THE INVENTION

The primary object of the present invention is to provide an adenovirus-based expression system capable of expressing a heterologous gene(s) in a host mammalian cell.

The present invention provides a novel, highly efficient, recombinant adenovirus expression system for expression of a heterologous gene(s) and/or gene product(s) in a mammalian cell. The recombinant adenovirus expression system of the invention was produced via homologous recombination between the novel vector of the invention co-transfected with the large fragment of the adenovirus-5 genome in 293 cells.

derivative thereof. Homologous recombination between these DNA fragments results in the production of a replication defective, recombinant adenovirus. The recombination reconstructs the adenovirus-5 genome by displacing the E1A and E1B protein coding regions with the plasmid vector cDNA.

In another embodiment of the invention, there is provided a recombinant adenovirus expression system for the receptor for thyrotropin-releasing hormone (TRH-R). The recombinant adenovirus, designated AdCMVmTRHR, circumvents difficulties encountered when using conventional transient or stable plasmid expression systems. Using this recombinant adenovirus (AdCMVmTRHR), TRH-Rs can be expressed in different mammalian cell types, including those resistant to transient transfection assay. Recombinant adenovirus, AdCMVmTRHR, was produced by homologous recombination between plasmid vector, designated pAdCMVmTRHR, i.e. the generic plasmid vector of the invention containing the gene coding TRH-R, co-transfected with the large fragment of adenovirus-5 d1309 genome. The versatility of using adenovirus mediated gene transfer and expression of TRH-Rs not only facilitates *in vitro* studies of TRH-R biology, but provides a valuable *in vivo* expression vector capable of extending TRH-R studies in animal model systems.

In a further embodiment of the present invention, infection of cultured fetal and adult rat cardiac myocytes *in vitro* and of adult cardiac myocytes *in vivo* was characterized using the recombinant adenovirus of the invention. The recombinant adenovirus, designated AdCMVCATgD, includes the chloramphenicol acetyltransferase (CAT) reporter gene driven by the cytomegalovirus (CMV) promoter. Plasmid vector pAdCMVCATgD i.e., generic plasmid vector of the present invention containing the gene encoding the bacterial CAT sequence, was co-transfected with the large fragment of the adenovirus-5 genome (3.6-100 map units). Homologous recombination between the plasmid vector and adenovirus fragment produced the recombinant adenovirus, designated AdCMVCATgD.

Virtually all fetal or adult cardiocytes expressed the CAT gene *in vitro* when infected with 1 plaque forming unit (pfu) of virus per cell. Using *in vitro* studies as a guide, recombinant virus AdCMVCATgD was introduced directly into adult rat

Fig. 12 is a graphic map showing the structure of plasmid vector pML-E1aEF-5778.

Fig. 13 is a graphic map of plasmid vector pAdCMVmTRHR used for the construction of recombinant adenovirus AdCMVmTRHR.

Fig. 14 is a graph showing a comparison of infection with AdCMVmTRHR and transfection with pAdCMVmTRHR on expression of TRH-Rs and *methyl*TRH responsiveness in six mammalian cell lines.

Fig. 15 is a graph showing TRH-induced desensitization and PMA-induced inhibition of the TRH response in AdCMVmTRHR-infected GHY, COS-1 and KB cells.

Fig. 16 is a graph showing *methyl*TRH-stimulated TRH-R internalization in AdCMVmTRHR-infected GHY, COS-1 and KB cells.

Fig. 17 is a graphic map of plasmid vector pAdCMVCATgD used in construction of recombinant adenovirus AdCMVCATgD.

Fig. 18(a) is a graph showing dosage and time dependent expression of adenovirus in fetal cardiocytes.

Fig. 18(b) is a graph showing dosage and time dependent CAT expression following infection by AdCMVCATgD in adult cardiocytes.

Fig. 19 is a graph showing distribution of CAT activity in cells of AdCMVCATgD injected hearts.

Fig. 20A is a graph showing CAT expression in the left ventricle 5 days following intracardiac injection of four doses of adenovirus [AdCMVCATgD; $6x10^6$, (n=4); $6x10^7$, (n=4); $6x10^8$, (n=3); and $2x10^9$, (n=2)].

Fig. 20B is a graph showing CAT expression over time in the left ventricle following injection of $6x10^7$ pfu of AdCMVCATgD virus.

Fig. 21 (a-f) is an immunohistochemical staining for CAT protein in adenovirus infected hearts.

Fig. 22 is a schematic showing the nucleotide sequence of plasmid vector pAdCMV-HS-Vector, as shown in Figs. 1(a) & 1(b).

Fig. 23 is a schematic showing the nucleotide sequence of another version of plasmid vector pAdCMV-HS-vector.

electrophoresis, identification of the fragment of interest by comparison of its mobility versus that of marker DNA fragments of known molecular weight, removal of the gel section containing the desired fragment, and separation of the gel from DNA. These procedures are generally well known. For example, see Lawn et al., 1981, *Nucleic Acids Res.*, vol. 9, pp. 6103-6114; and Goeddel et al., 1980, *Nucleic Acids Res.*, vol. 8, p. 4057, which disclosures are hereby incorporated by reference.

The term "expression" may be characterized as follows: A cell is capable of synthesizing many proteins. At any given time, many proteins which the cell is capable of synthesizing are not being synthesized. When a particular polypeptide, coded for by a given gene, is being synthesized by the cell, that gene is said to be expressed. In order to be expressed, the DNA sequence coding for that particular polypeptide must be properly located with respect to the control region of the gene. The function of the control region is to permit the expression of the gene under its control.

The term "southern blot analysis" is a method by which the presence of DNA sequences in a restriction endonuclease digest of DNA or DNA-containing composition is confirmed by hybridization to a known, labeled oligonucleotide or DNA fragment. Southern analysis typically comprises electrophoretic separation of DNA digests on agarose gels, denaturation of the DNA after electrophoretic separation, and transfer of the DNA to nitrocellulose, nylon, or another suitable membrane supports for analysis with a radiolabeled, biotinylated or enzyme-labeled probe as described in sections 9.37-9.52 of Sambrook et al., supra, which disclosure is hereby incorporated by reference.

The term "northern analysis" is a method used to identify RNA sequences that hybridize to a known probe such as an oligonucleotide, DNA fragment, cDNA or fragment thereof, or RNA fragment. The probe is labeled with a radioisotope such as ^{32}P, or by biotinylation, or with an enzyme. The RNA to be analyzed is usually electrophoretically separated on an agarose or poly-acrylamide gel, transferred to nitrocellulose, nylon, or other suitable membrane, and hybridized with the probe, using standard techniques well known in the art, such as those described in sections

compatible for ligation. To blunt the ends, the DNA is treated in a suitable buffer for at least 15 minutes at 15° C, with about 10 units of the Klenow fragment of DNA polymerase I or T4 DNA polymerase in the presence of the four deoxyribonucleotide triphosphates. The DNA is then purified by phenolchloroform extraction and ethanol precipitation. The DNA fragments that are to be ligated together are put in solution in about equimolar amounts. The solution will also contain ATP, ligase buffer, and a ligase such as T4 DNA ligase at about 10 units per 0.5 g of DNA. If the DNA is to be ligated into a vector, the vector is first linearized by digestion with the appropriate restriction endonuclease(s). The linearized fragment is then treated with bacterial alkaline phosphatase, or calf intestinal phosphatase to prevent self-ligation during the ligation step.

"Preparation" of DNA from cells means isolating the plasmid DNA from a culture of the host cells. Commonly used methods for DNA preparation are the large and small scale plasmid preparations described in sections 1.25-1.33 of Sambrook et al., supra, which disclosure is hereby incorporated by reference. After preparation of the DNA, it can be purified by methods well known in the art such as that described in section 1.40 of Sambrook et al., supra, which disclosure is hereby incorporated by reference.

This invention achieves significantly enhanced *in vitro* and *in vivo* expression levels of heterologous gene(s) by inserting into a host mammalian cell the adenovirus expression system or plasmid vector of the invention, containing foreign cDNA encoding the heterologous gene(s) under the transcriptional control of DNA fragments derived from the mouse cytomegalovirus (CMV) immediate early gene regulatory sequences. It is understood that the CMV immediate early promoter can be combined with enhancer elements isolated from other transcriptional units to increase expression efficiency.

The recombinant adenovirus expression system and plasmid vector include at least one cDNA insertion site(s) i.e., restriction site(s) for cloning a selected heterologous gene(s). Other important features of the adenovirus expression system and plasmid vector of the invention include a highly efficient eukaryotic splicing

nucleotide sequence from nucleotide 2800-5776, which serves as the region for homologous recombination.

To obtain efficient expression of the heterologous gene(s), a eukaryotic promoter must be present in the plasmid vector and recombinant adenovirus expression system. It is understood that any known eukaryotic promoter can be utilized in the plasmid vector and/or recombinant adenovirus expression system of the invention provided the promoter is capable of expressing the heterologous gene(s). The promoter used herein, preferably, is the mouse cytomegalovirus-1 early promoter, or an effective expression promoting fragment thereof. For an example of the CMV promoter, see U.S. Patent No. 4,963,481 to Jean P. deVilliers, which disclosure is hereby incorporated by reference. The use of the mouse CMV promoter is of broad utility because this promoter has a very broad host range and functions with superior strength and efficiency in a wide variety of cell lines tested.

The presence and position of the splicing elements with respect to the cDNA are important to overall processing efficiency, as is the choice of splicing elements. In the present invention, a hybrid splice donor and acceptor was used which yielded a highly efficient processing activity compared to the more common splice element used in other systems i.e., the SV40 small T splice site. By inserting the cDNA downstream of the splice elements, we are coupling the splice elements to the downstream 3' processing site generating a terminal exon. Use of a demonstrably efficient poly(A) site maximizes efficiency of the expression system. This allows efficient conversion of pre-mRNA into mRNA and allows the system to take full advantage of the high level of expression generated by the CMV promoter.

Any of the conventional cloning methods for insertion of the gene and/or gene fragment(s) into the plasmid vector can be used to ligate the promoter and the other control elements into specific sites within the plasmid vector. Accordingly, heterologous gene sequence(s) containing those regions coding for the gene(s) can be ligated into the plasmid vector at a specific restriction site in relation to the promotor and control elements so that when either the recombinant adenovirus or plasmid vector

chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard techniques.

The protein(s) encoded by the heterologous gene(s) inserted into the plasmid vector and recombinant adenovirus expression system can comprise any known protein, including; growth hormone, human growth hormone (HGH), des-N-methionyl human growth hormone, bovine growth hormone, parathyroid hormone, thyroxine, insulin A-chain, insulin B-chain, proinsulin, relaxin A-chain, relaxin B-chain, prorelaxin, follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), leutinizing hormone (LH), glycoprotein hormone receptors, calcitonin, glucagon, factor VIII, an antibody, lung surfactant, urokinase, streptokinase, human tissue-type plasminogen activator (t-PA), bombesin, factor IX, thrombin, hemopoietic growth factor, tumor necrosis factor-alpha and -beta, enkephalinase, human serum albumin, mullerian-inhibiting substance, mouse gonadotropin-associated peptide, β-lactamase, tissue factor protein, inhibin, activin, vascular endothelial growth factor, integrin receptors, thrombopoietin, protein A or D, rheumatoid factors, NGF-β platelet-growth factor, transforming growth factor; TGF-alpha and TGF-beta insulin-like growth factor-I and -II, insulin-like growth factor binding proteins, CD-4, DNase, latency associated peptide erythropoietin, osteoinductive factors, interferon, alpha, - beta, and -gamma, colony stimulating factors (CSFs), M-CSF, GM-CSF, and G-CSF, interleukins (ILs), IL-1, IL-2, IL-3, IL-4, IL-6, IL-7, superoxide dismutase; viral antigens; HIV envelope proteins GP120 and GP140, immuno globulins, and fragments of the above listed proteins.

The following Examples are provided to further illustrate the present invention.

Example I

Construction of pGEM2AdCMV

Plasmid pBstSK+ (0-353) contains adenovirus-5 sequence from nt 0-353 inserted into pBstSK+ vector at the *Eco*RI site and the *Sst*II site (which has been lost by blunt end ligation). These sequences are required viral elements which include the origin for DNA replication and the viral packaging sequence. The CMV

Example IV
Construction of pAdCMVdHCatgD

One of the three *Hind*III restriction sites in pAdCMVCatgD (Figure 6) was deleted by partial *Hind*III digestion and filling by Klenow large fragment of DNA polymerase followed by plasmid circularization and ligation. This allowed removal of the CAT sequence and the poly(A) site by *Hind*III digestion, with the retention of promoter and splicing sequences. A 1100 bp of E1B sequence was deleted.

Example V
Construction of pAdCMVCatgDNeo(-)

The unique restriction site *Not*I located at position 361 can be used to insert any additional gene of interest. As a test construct a *Not*I fragment from pPYNeo was isolated which contained the Neomycin resistance gene driven by the polyoma promoter and using the SV40 splicing and polyadenylation elements. This strategy resulted in the introduction of the Neo gene into the vector in two orientations relative to the direction of CAT gene expression (+) and (-). Both of these constructs were used in virus constructions, however, only the AdCMVCatDNeo(-) virus has been isolated to date.

Example VI
Construction of pAdCMVTRHrE3

Using vector pAdCMVdHCatgD (Figure 6), cDNA for thyrotropin releasing hormone receptor (which contains the adenovirus E2 poly(A) site) was inserted directly into the *Hind*III digested vector to construct vector pAdCMVTRHrE3 (Figure 9).

2. Construction of AdCMVmTRHR:

The parent plasmid, pAdCMVmTRHR, was constructed by inserting a 1.2 kb *Eco*RI-*Not*I fragment containing the protein-coding region of the mouse TRH-R cDNA, nucleotides 233-1462 of plasmid pBSmTRHR (Straub et al., 1990, *Proc. Natl. Acad. Sci. U.S.A.*, vol. 87, pp. 9514-9518, which disclosure is hereby incorporated by reference), into plasmid pGEM2-L3-114 at the *Eco*RI-*Bam*HI site. After digesting with *Eco*RI and using the Klenow fragment of DNA polymerase I to make blunt DNA ends, *Hind*III linkers were ligated and a 1.4 kb *Hind*III fragment containing mouse TRH-R cDNA and the adenovirus E2 poly(A) signal sequence was isolated and inserted into the *Hind*III site of the pAdCMV-HS-Vector (i.e., expression plasmid of the present invention) which contains the left end replication and packaging elements of adenovirus, the cytomegalovirus-1 promoter and splicing elements from plasmid pML-IS Cat (Huang et al., 1990, *Nucleic Acids Res.*, vol. 18, pp. 937-947, which disclosure is hereby incorporated by reference). Following verification of the plasmid by restriction site mapping and transient transfection of pAdCMVmTRHR into COS-1 cells to demonstrate TRH-R expression, the virus AdCMVmTRHR was constructed by overlap recombination as described by Tantravahi et al., 1993, *Mol. Cell. Biol.*, vol. 13, pp. 578-587, which disclosure is hereby incorporated by reference. All transfections were carried out in human embryonic kidney cells transformed with the E1 region of adenovirus type 5 according to the procedure of Graham et al., 1977, *J. Gen. Virol.*, vol. 36, pp. 59-72, which disclosure is hereby incorporated by reference. Following plaque purification, virus was grown in 293 cells in suspension cultures as described by Antravahi et al., 1993, *Mol. Cell. Biol.*, vol. 13, pp. 578-587, which disclosure is hereby incorporated by reference. The entire sequence coding for the adenovirus E1a gene was removed as well as the 5' 1.8 kb of the E1b gene. Co-transfection of pAdCMVmTRHR with the large fragment of adenovirus (3.8-100 map units) into 293 cells resulted in production of recombinant virus AdCMVmTRHR.

9514-9518, which disclosure is hereby incorporated by reference). For GHY, AtT-20 and C6 cells, incubation with plasmid and DEAE dextran was for 0.5 hours at 4°C and no chloroquine or dimethylsulfoxide was added (Fujimoto et al., *Endocrinology*, vol. 130, pp. 1879-1884, which disclosure is hereby incorporated by reference). Cells were studied 48 to 72 hours after transfection, which are times of maximum TRH-R expression.

5. Measurement of TRH-R number:

Binding of 0.1 to 7.5 nM [^{3}H]*methyl*TRH, an analog of higher affinity and potency than TRH (Vale et al., 1971, *Endocrinology*, vol. 89, pp. 1485-1488, which disclosure is hereby incorporated reference), to intact cells was measured as described by Gershengorn, M.C., 1978, *J.Clin. Invest.*, vol. 62, pp. 937-943, which disclosure is hereby incorporated by reference. Binding isotherms were fitted and dissociation constants (K_ds) and receptor numbers (one-to-one stoichiometry of *methyl*TRH and receptor) were obtained with the INPLOT program (Graphpad). Receptor number was calculated using the following equation: fractional occupancy = $1 / [1 + (K_d/L)]$. Receptor number is given assuming that all cells in the population are expressing equal numbers of TRH-Rs. This appears to be the case with infections using 300 AdCMVmTRHR particles per cell (not shown).

6. Measurement of TRH response:

Infected or transfected cells were labelled for 24 hours with [^{3}H]*myo*-inositol, stimulated with TRH or *methyl*TRH in a balanced salt solution containing 10 mM LiCl and [^{3}H]IPs were measured as described by Imai et al., 1987, *Methods. Enzymol.*, vol. 141, pp. 100-101, which disclosure is hereby incorporated by reference.

7. Measurement of desensitization and inhibition by PMA:

Cells were incubated in medium with serum containing *myo*-[^{3}H]inositol (1 μCi/ml) for 24 hours prior to infection and studied 16 to 24 hours after infection.

AdCMVmTRHR (Fig. 13) was similar to that used in the construction of the plasmid vector constructs of the invention (Figs. 1(a) & 1(b)). With reference to Figure 13, there is shown plasmid vector pAdCMVmTRHR, which was used to produce recombinant adenovirus AdCMVmTRHR. Turning to Figure 13, the left end of the adenovirus starts at position 1. The adenovirus sequence from nucleotide 1-353 contains the origin of replication and the viral packaging sequence. The adenovirus sequence from 354-2800 is deleted and replaced with the CMV-1 promoter, splice elements, the protein coding region of the mouse TRH-R cDNA sequence and the E2 poly(A) site. The left end adenovirus sequence from nucleotides 2800-5776 serve as the region for homologous recombination.

The novelty of AdCMVmTRHR as a vector for expression of TRH-Rs and its advantage over transfection are illustrated in Figure 14. With reference to Figure 14, the levels of TRH-R expression (upper panel) and *methyl*TRH stimulation of [^{3}H]IP formation (lower panel) were measured as previously described. The data in the (upper panel) are presented as number of receptors per cell assuming that all cells express equal numbers of TRH-Rs. The bars in both panels represent the mean ±SD of triplicate determinations in a representative experiment that was performed 3 times. In these experiments the plasmid vector used for virus construction, and expression of TRH-Rs after infection with AdCMVmTRHR and after transfection with pAdCMVmTRHR, were compared in HeLa cells, rat pituitary tumor GHY cells, mouse pituitary tumor AtT-20 cells, rat glioma C6 cells and monkey kidney CV1 and COS-1 cells. These cell lines were chosen because they represent a wide variety of cell types which do not express TRH-Rs. That is, HeLa cells were studied because they are readily infected with adenovirus. GHY cells were studied because they are a subclone of the cells in which endogenous TRH-Rs have been most well-studied. COS-1 cells were studied because they are a commonly used, transformed cell line that permits high levels of expression during transient assays.

TRH-Rs expressed on the surface of these cells after infection with AdCMVmTRHR bound *methyl*TRH with the same affinity as native TRH-Rs on mouse pituitary cells (Gershengorn et al., 1978, *J. Clin. Invest., vol.* 62, pp. 937-943, which

all cell types after infection by AdCMVmTRHR than after transfection. However, there was no correlation between the magnitude of the *methyl*TRH response and the number of TRH-Rs when comparing different cell types. For example, *methyl*TRH stimulation of IP formation was greater in AtT-20 cells which expressed TRH-Rs at a lower number than in HeLa cells with a greater number of TRH-Rs. One explanation for this observation may be that there are differences in post-receptor components of the signal transduction cascades within these different cell types. Another finding was that the magnitude of response to *methyl*TRH in COS-1 cells was greater after infection than after transfection even though the total number of receptors was similar. This may be because all COS-1 cells expressed a maximally effective number of TRH-Rs after AdCMVmTRHR infection, whereas only a fraction of the transfected cells were expressing maximally effective numbers of TRH-Rs because infection is more efficient than transfection.

In rat GH_3 pituitary cells naturally expressing TRH-Rs, the TRH response is rapidly desensitized (Perlman et al., 1991, *Endocrinology, vol.* 129, pp. 2679-2686, which disclosure is hereby incorporated by reference). This effect occurs prior to any decrease in the number of TRH-Rs ("down-regulation") (Gershengorn, M. C., 1978, *J. Clin. Invest., vol.* 62, pp. 937-943; and Hinkle et al., 1975, *Biochemistry, vol.* 14, pp. 3845-3851, which disclosures are hereby incorporated by reference. This response to TRH is also blunted in GH_3 cells preincubated with phorbol esters, such as PMA, which activate protein kinase C (Drummong, A. H., 1986, *J. Exp. Biol., vol.* 124, pp. 337-358, which disclosure is hereby incorporated by reference). Evidence, however, is presented that these two effects are distinct and suggested that TRH-induced desensitization is not mediated primarily by protein kinase C (Perlman et al., 1991, *Endocrinology,* vol. 129, pp. 2679-2686, which disclosure is hereby incorporate by reference). Although the molecular mechanisms of TRH-induced desensitization and of PMA-induced inhibition of the TRH response have not been elucidated, it is likely that they are mediated by receptor phosphorylation (Lefkowitz et al., 1992, *Cold Spring Harbor Symp. Quant. Biol.,* vol. 57, pp. 127-134, which disclosure is hereby incorporate by reference). Because different cell types contain

Rapid internalization is another process that many GPCRs, including TRH-Rs (Nussenzveig et al., 1993, *J. Biol. Chem.*, vol. 268, pp. 2389-2392; and Hinkle, P. M., 1989, *Ann. N. Y. Acad. Sci.*, vol. 553, pp. 176-187, which disclosures are hereby incorporated by reference), undergo after binding (Dohlman et al., 1991, *Annu. Rev. Biochem.*, vol. 60, pp. 653-688, which disclosure is hereby incorporated by reference). To determine whether TRH-R internalization is cell type specific, we measured internalization of bound *methyl*TRH in three AdCMVvTRHR-infected cell lines which displayed differences in TRH-induced desensitization or PMA-induced inhibition of the TRH response, or both. Internalization in cell lines that do and do not exhibit rapid desensitization induced by TRH was measured because it has been controversial whether these two processes are related. Figure 16 illustrates that internalization of *methyl*TRH-bound TRH-Rs was faster in AdCMVmTRHR-infected GHY cells than in COS-1 cells and KB cells but that the fraction of receptors internalized after 60 minutes was similar in all three cell types. After 60 minutes, 64±7.0%, 62±2.1%, and 71±2.6% of TRH-Rs were internalized in AdCMVmTRHR-infected GHY, COA-1 and KB cells, respectively. With reference to Figure 16, internalization of TRH-Rs was measured as previously described. The data represent mean ±SD of triplicate determinations in a representative experiment performed twice. In these three cell lines, agonist-induced internalization of TRH-Rs exhibited small kinetic differences but the extent of internalization after 60 minutes, the time at which measured desensitization were similar.

A number of aspects of GPCR biology may vary when receptors are expressed in different cell types. For example, the same GPCR may activate different signal transduction pathways when expressed in different cell types (Milligan et al., 1993, *Trends Pharmacal. Sci.*, vol. 553, pp. 176-187, which disclosure is hereby incorporated by reference). Agonist-induced desensitization, which is a process that commonly accompanies activation of GPCRs, appears to be mediated by a conserved set of intracellular regulatory proteins including protein kinases and arrestin-like proteins (Lefkowitz et al. 1993, *Adv. Second Messenger Phosphoprotein Res.*, vol. 28, pp. 1-9; and Lefkowitz, R. J., 1993, *Cell*, vol. 74, pp. 409-412, which disclosures are hereby

Example VIII

Quantitative Determination of Adenovirus-Mediated Gene Delivery to Rat Cardiac Myocytes *In Vitro* and *In Vivo*

1. Isolation and culture of Rat Cardiac Myocytes:

Primary fetal cardiac myocytes were prepared from fetal day 20 Sprague-Dawley rats (Taconic Farms) by modification of the protocol of de Carvalho et al., 1992, *Circ. Res.*, vol. 70, pp. 733-742, which disclosure is hereby incorporated by reference. Cardiac cells were preplated for 1 hour in order to remove fibroblasts. $1.8X10^6$ cells were then plated per 25mm tissue culture dishes (Corning) in heart medium (Hank's salt solution supplemented with MEM Vitamin Stock, MEM amino acids, MEM non-essential amino acids, L-Glutamine (2mM), 1% Glycine, 2% Hypoxanthine, 1% Penn-Strep, $NaHCO_3$) with 10% fetal bovine serum (Hyclone). Primary adult cardiac myocytes were prepared from the hearts of 200g female Sprague-Dawley rats (Taconic Farms) according to the protocol of White et al. 1993, *Biophys. J.*, vol. 65, pp. 196-204, which disclosure is hereby incorporated by reference. 2.4×10^5 cells were plated in heart medium per 60mm dish coated with 20 ug/ml of laminin (Boehringer Mannheim). Cells were maintained in culture at 37°C, 5% CO_2. Cell culture medium was changed every other day for the duration of the assay.

2. Virus production:

Virus plaguing and the preparation of viral stocks were performed on 293 monolayer cells as described by Tantravahi et al., 1993, *Mol. Cell. Biol.*, vol. 13, pp. 578-587, which disclosure is hereby incorporated by reference.

3. Infection of cardiac myocytes:

Forty eight hours after plating, fetal myocytes were infected with AdCMVCATgD at 0.01, 0.1, 1, 10 pfu/cell. The adult cells were infected with the same doses immediately after plating. AdCMVCATgD (10^{10} pfu/ml) was diluted in heart media without added serum. One ml of media + virus was added to each 60mm

were then washed in dH_2O and mounted with gelvatol (Airvol, Air Products and Chemicals, Inc.).

5. CAT assays from myocytes:

At each time point, infected cardiac myocytes were harvested according to the protocol of Ausbel et al., 1989, *Current Protocols in Molecular Biology*, Wiley, New York, which disclosure is hereby incorporated by reference. The amount of protein in the supernatant was measured by Bradford assay using bovine serum albumin (BSA) as the standard (BioRad). CAT assays were performed on 10 μg of total protein. When the amount of CAT activity was greater than 70% and out of the linear range, supernatants were diluted in 0.1 mg/ml BSA. CAT assays were done by TLC according to the method of Kitsis et al., 1993, in *Methods in Molecular Genetics*, ed. Adolph, K.W., Academic Press, Inc., New York, Vol. 1, pp. 374-392, which disclosure is hereby incorporated by reference, incubating for 2 hours at 37°C.

6. DNA and virus injections *in vivo*:

10 μg of CMV CAT plasmid DNA in 50μl PBS was injected into the apex of the left ventricle of 200g female Sprague Dawley rats as described by *Kitsis et. al.*, 1993, in *Methods in Molecular Genetics*, ed. Adolph, K.W., Academic Press, Inc., New York, vol. 1, pp. 374-392, which disclosure is hereby incorporated by reference. For the adenovirus injections, 6×10^6 to 6×10^8 pfu in 50 μl PBS were injected, 2×10^9 pfu were injected undiluted in a volume of 50 μl.

7. CAT assays on tissue:

At indicated times following injection, hearts were removed, rinsed in PBS and weighed. For the spatial distribution experiment the hearts were then sectioned into seven roughly equivalent slices. Each slice was then homogenized using a Tissumizer (Tekmar) in a volume of 0.5 mls buffer (1M Gly gly pH 7.8, 150mM $MgSO_4$, 500mM EGTA pH 8.0, 1M DTT) for 20 sec. For the dosage, and time course experiments the hearts were homogenized the same way but in a volume equal to 0.5g

dilutions of cell extracts were made to maintain assays in the linear range of the CAT assay.

CAT activity was easily detected at the earliest measured time point (4 hours), was near maximal by 48 hours, and was maintained at stable levels through the remainder of the experiment (a total of 167 hours). A dose-dependent increase was maintained over a range of hour logs of virus input throughout much of the time course. The same basic extent and level of infection and expression was found in adult cardiocytes (Figure 16(b)) when infected under similar conditions. However, the duration of study was shortened to 48 hours due to the difficulty in maintaining healthy differentiated adult cardiac myocytes in culture, independent of virus infection. Based on these assays, the sensitivity of the AdCMVCATgD CAT assay, and the levels of activity resulting from these infections, it was redacted that CAT expression could be reliably detected in as few as 10 infected cells.

At each dose of virus, the percentage of fetal cells which were expressing CAT was determined by immunostaining coverslips of infected fetal cardiocytes 18 and 48 hours post infection. Mock-infected cells show no staining, but cells infected with increasing doses of virus show a proportional increase in the number of cells infected, with 1 pfu/cell (100 particles) resulting in virtually 100% of the cells being stained (data not shown). The virus infection included both myocytes and the small proportion of nonmyocyte fibroblasts (<5%) which remained in the culture following initial myocyte purification (data not shown). Similar results were obtained with adult cardiac myocytes. At an infection of 1 pfu or greater, 100% of the rod-shaped adult myocytes stained positive with an anti-CAT antibody. This was true at both 4 and 48 hours. Myocytes which were rounded up also stained positive for CAT, and sarcomeric myosin heavy chain, and excluded trypan blue (data not shown).

Adenovirus mediated gene transfer offers advantages to transient transfection assays when using cultured myocytes. The quantitative advantages of using AdCMVCATgD *in vitro* was examined to determine whether it could be extended to *in vivo* studies. 6×10^7 pfu of AdCMVCATgD virus were injected into adult rat hearts in a volume of 50 µl. A parallel injection of 10 µg of the plasmid pAdCMVCATgD was

CAT antigen at all doses of virus. Three doses of viral input are shown. A,B=$6X10^6$; C,D=$6X10^7$; E,F=$2X10^9$. Photographs of tissue sections were taken under Differential Interference Contrast (DIC) microscopy). A,C,E; Bar = 1mm, B,D,F; Bar = .05mm. CAT positive cells are stained brown for peroxidase reaction. All sections are counterstained with hematoxylin. In many regions, virtually 100% of myocytes stain positive. Positive cells include both myocytes and nonmyocytes, although it appears that the proportion of myocytes infected exceeds that of non-myocytes. A substantial number of inflammatory cells were seen (See Figure 21(d) & 21(e)). The nature of this inflammatory response is currently under investigation but does not appear to correlate with the amount of introduced virus. The intensity of peroxidase staining appeared to increase with increasing viral dose. It appears that the lowest dose of virus ($6X10^6$) resulted in a lower intensity of CAT antigen/cell as well as reduced number of infected cells. At higher doses of virus, both an increased number of cells and an increased amount of CAT/cell were obtained.

Cardiac myocytes appear to be ideally suited for the use of adenovirus mediated gene transfer. Transient transfection of fetal cardiocytes under optimized conditions traditionally results in 10-20% of the cells being transfected. Adenovirus can infect virtually 100% of cells and does not require the use of damaging treatments such as electroporation which generally kills a large number of the cells in the culture. Clearly, fetal cardiocytes possess viral receptors in numbers do not present a limitation to use of adenovirus vectors in rat cardiocytes. With adenovirus infection, there is no apparent effect on cell viability or morphology at the pfu ratios tested here. In addition, adenovirus infections also provide an efficient means of gene transfer into adult cells which has not been possible using conventional transfection strategies (Kirshenbaum et al., 1993, *J. Clin. Invest., vol.*, 92, pp. 381-387, which disclosure is hereby incorporated by reference). A recent report of adenovirus infection of adult rat cardiocytes (Kirshenbaum et al., 1993, *J. Clin. Invest.*, vol. 92, pp. 381-387, which disclosure is hereby incorporated by reference) reported 90% infection at a dose of 10^3 pfu/cell. Based on the results of the present invention, it is not necessary to use such a high dose of virus. Because of the efficient CAT expression system, the viral dose

obtained in accordance with the present invention and those of Lemarchand *et. al.*, 1993, *Circ. Res.*, vol. 72, pp. 1132-1138, which disclosure is hereby incorporated by reference, demonstrate a rather transient pattern of expression. It may be that in order to generate long-term expression it will be necessary to introduce the virus into neonates, as has been suggested by Strattford-Perricaudet *et al.*, 1992, *J. Clin. Invest.*, vol. 90, pp. 626-630, which disclosure is hereby incorporated by reference. Studies are currently underway to examine the effect of various routes of infection, tissue distribution and immune response to this virus *in vivo*. However, it is apparent that adenovirus mediated gene transfer in the heart is extremely efficient and should be a very useful tool for the introduction of genes into cardiac myocytes.

It should be understood, that the foregoing embodiments are provided for purpose of illustration only and, not limitation, and that all such modifications or changes which occur to persons skilled in the art are deemed to be within the spirit and scope of the present invention.

8. The vector according to Claim 1, wherein said vector comprises the nucleotide sequence as substantially shown in Figure 22 (Seq.Id.No.1).

9. The vector according to Claim 1, wherein said vector further comprises a separate site for insertion of a second transcription unit.

10. A method of producing a recombinant adenovirus expression vector for expression of a heterologous gene(s) and/or gene product(s) in a host cell capable of being infected by said adenovirus, comprising:

a) preparing the vector according to Claim 1;

b) co-transfecting said vector with an adenovirus-5 genome in 293 cells, under conditions which facilitate homologous recombination between said vector and adenovirus-5, thereby producing a recombinant adenovirus; and

c) isolating the recombinant adenovirus.

11. A recombinant adenovirus expression vector produced according to the method of Claim 10.

12. A host cell line or animal infected by the recombinant adenovirus expression vector according to Claim 11.

13. A unicellular host transformed by the vector according to Claim 1.

14. A method for producing a selected protein, comprising, culturing a host which has been infected with a recombinant adenovirus vector according to Claim 11.

15. A method for producing a selected protein, comprising culturing a transformed host which has been transformed with a vector according to Claim 1.

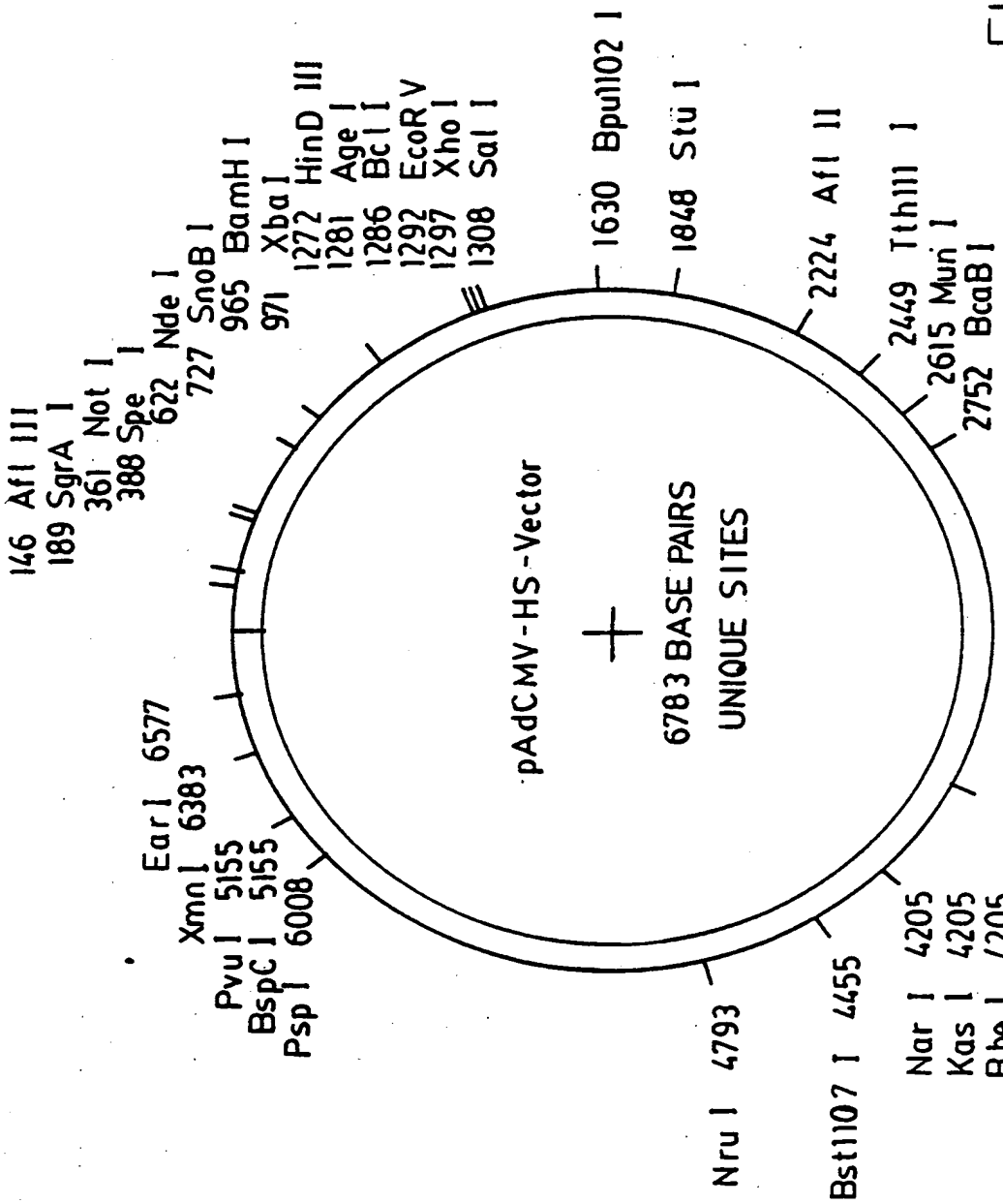
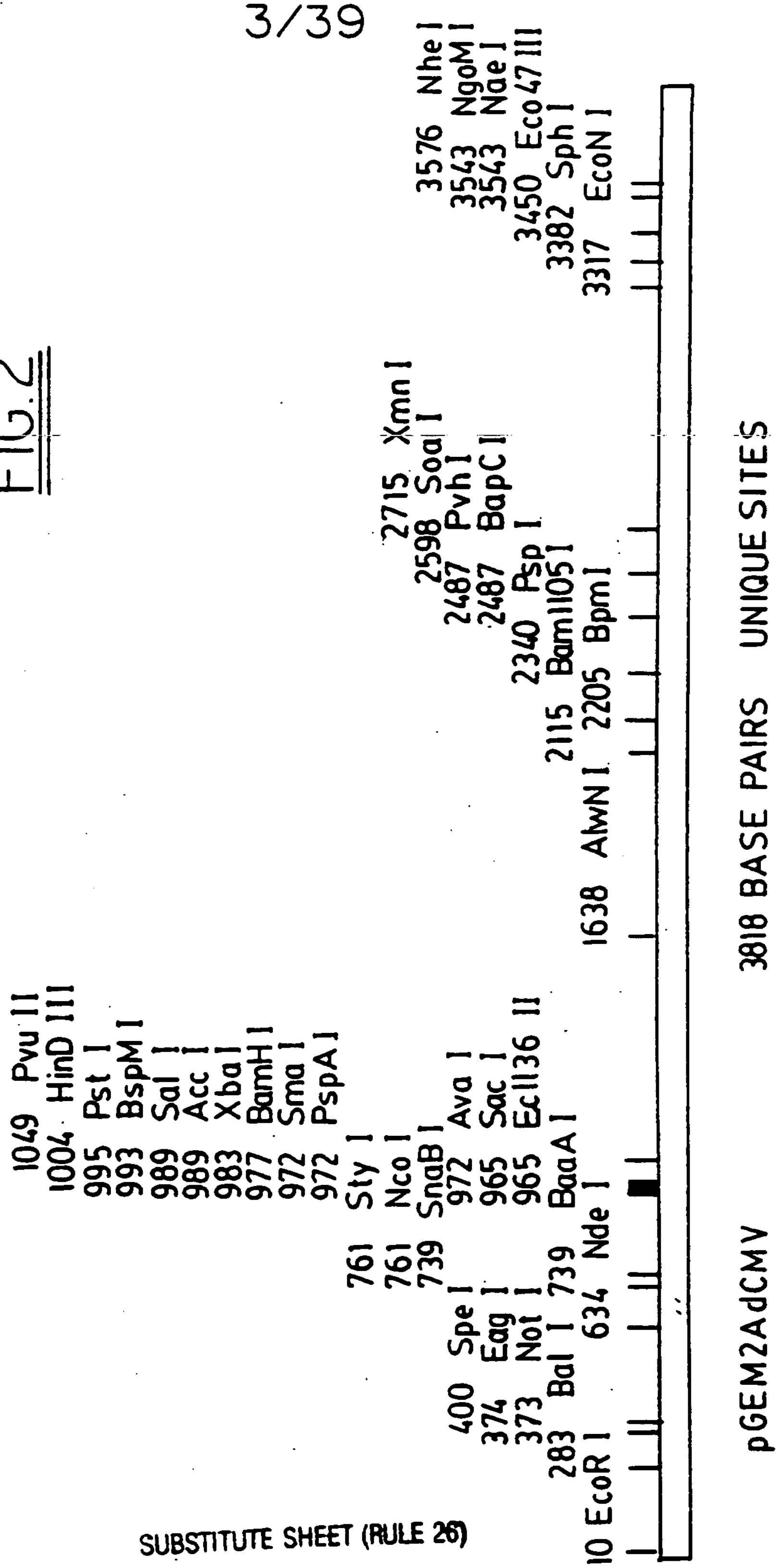

pAdCMV-HS-Vector
6783 BASE PAIRS
UNIQUE SITES
146 Afl III
189 SgrA I
361 Not I
388 Spe I
622 Nde I
727 SnoB I
965 BamH I
971 Xba I
1272 HinD III
1281 Age I
1286 Bcl I
1292 EcoR V
1297 Xho I
1308 Sal I
1630 Bpu1102 I
1848 Stu I
2224 Afl II
2449 Tth111 I
2615 Mun I
2752 BcaB I
3877 BstE11
4205 Bbe I
4205 Kas I
4205 Nar I
4455 Bst1107 I
4793 Nru I
5155 Psp I
5155 BspC I
6008 Pvu I
6383 Xmn I
6577 Ear I

FIG.1A

FIG. 2


10 EcoR I
283 Bal I
373 Not I
374 Eag I
400 Spe I
634
739 Nde I
739 SnaB I
761 Nco I
761 Sty I
965 BaaA I
965 Ecl136 II
965 Sac I
972 Ava I
972 PspA I
972 Sma I
977 BamH I
983 Xba I
989 Acc I
989 Sal I
993 BspM I
995 Pst I
1004 HinD III
1049 Pvu II
1638 AlwN I
2115
2205 Bpm I
2340 Bam1105 I
2487 Psp I
2487 BapC I
2598 Pvh I
2715 Soa I
Xmn I
3317 EcoN I
3382 Sph I
3450 Eco47 III
3543 Nae I
3543 NgoM I
3576 Nhe I
pGEM2AdCMV
3818 BASE PAIRS UNIQUE SITES

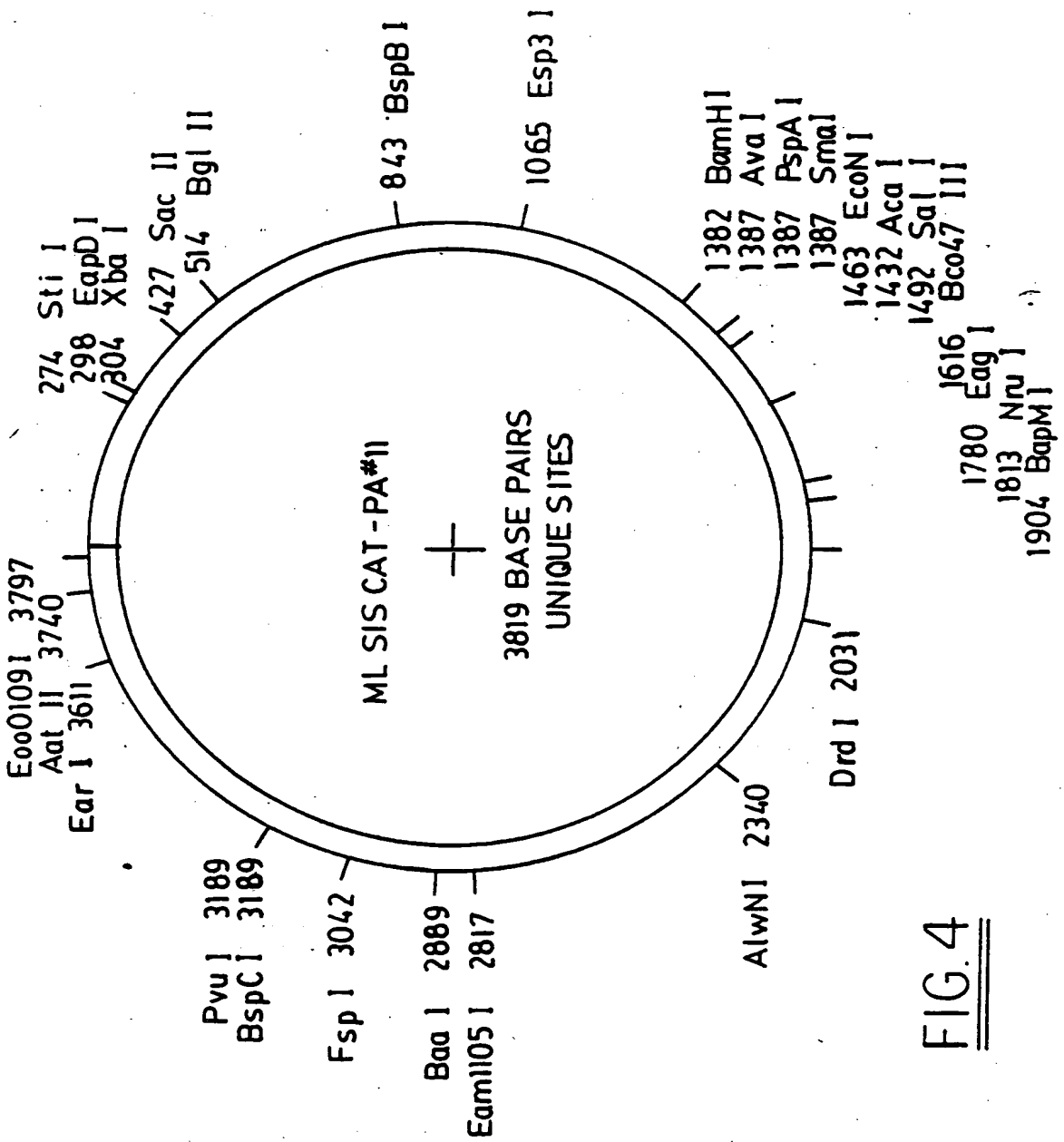

ML SIS CAT-PA#11
3819 BASE PAIRS
UNIQUE SITES
843 BspB I
1065 Esp3 I
1382 BamH I
1387 Ava I
1387 PspA I
1387 Sma I
1463 EcoN I
1432 Aca I
1492 Sal I
1616 Bco47 III
1780 Eag I
1813 Nru I
1904 BapM I
2031 Drd I
2340 AlwN I
2817 Eam1105 I
2889 Baa I
3042 Fsp I
3189 Pvu I
3189 BspC I
3611 Ear I
3740 Aat II
3797 Eco0109 I
274 Sti I
298 EapD I
304 Xba I
427 Sac II
514 Bgl II

FIG.4

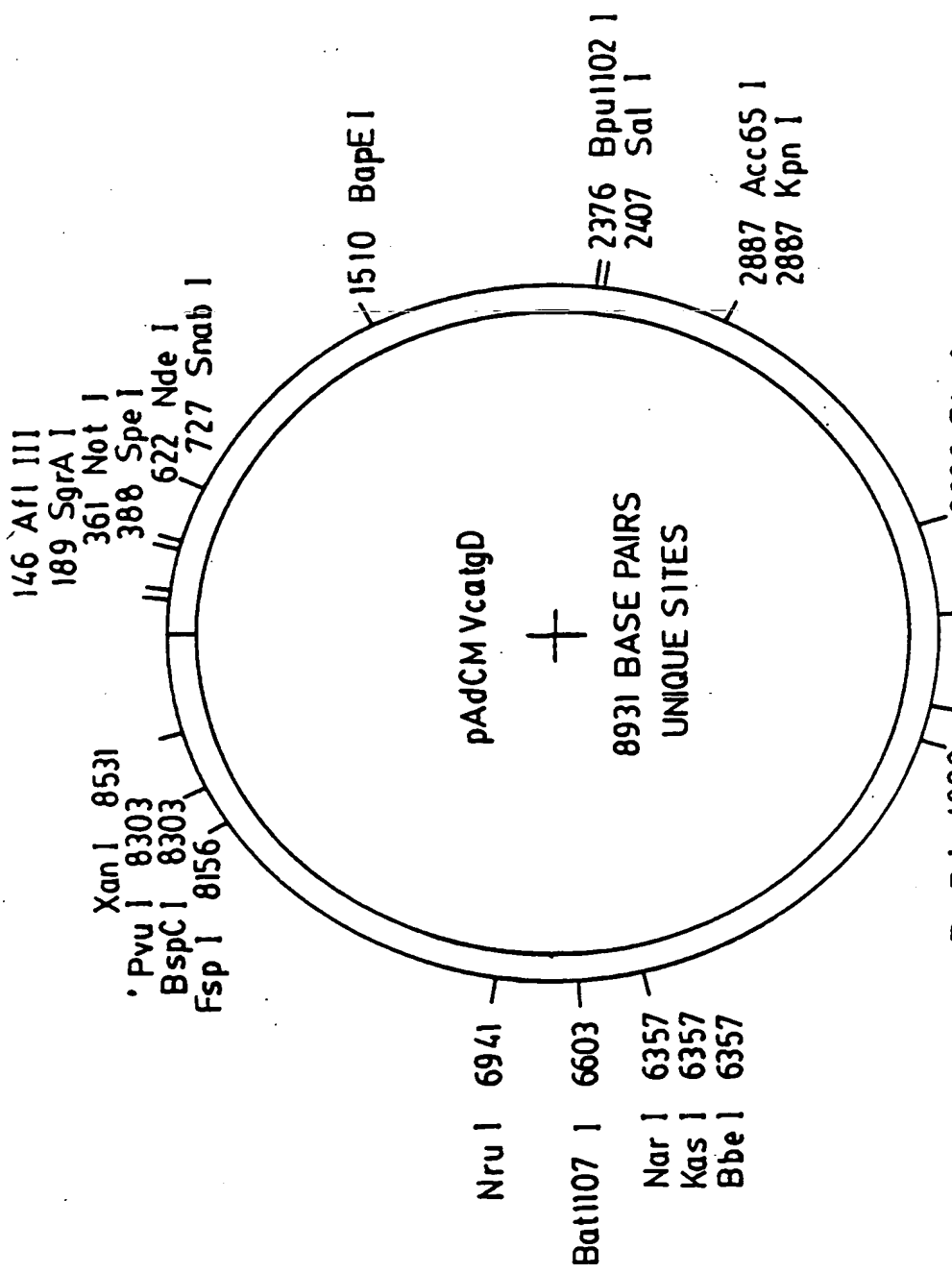

pAdCM VcatgD
8931 BASE PAIRS
UNIQUE SITES
1510 BapE I
2376 Bpu1102 I
2407 Sal I
2887 Acc65 I
2887 Kpn I
3996 Stu I
4372 Af1 II
BsaB I 4900
Mun I 4763
Nru I 6941
Bat1107 I 6603
Nar I 6357
Kas I 6357
Bbe I 6357
Xan I 8531
Pvu I 8303
BspC I 8303
Fsp I 8156
146 Af1 III
189 SgrA I
361 Not I
388 Spe I
622 Nde I
727 Snab I

FIG.6

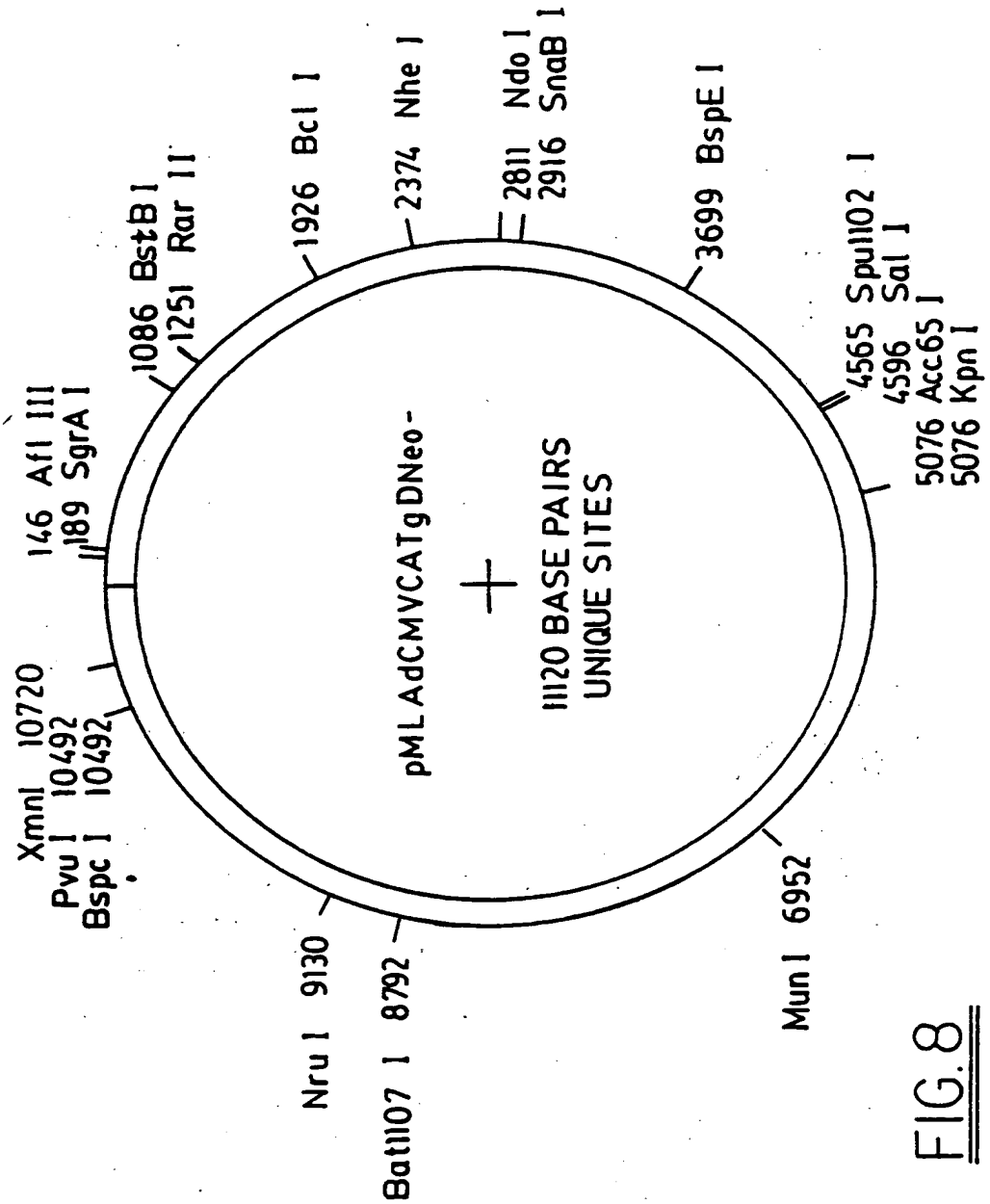

pMLAdCMVCATgDNeo-
11120 BASE PAIRS
UNIQUE SITES
146 Afl III
189 SgrA I
1086 BstB I
1251 Rar II
1926 Bcl I
2374 Nhe I
2811 Ndo I
2916 SnaB I
3699 BspE I
4565 Spu1102 I
4596 Sal I
5076 Acc65 I
5076 Kpn I
6952 Mun I
8792 Bat1107 I
9130 Nru I
10492 Bspc I
10492 Pvu I
10720 Xmn I

FIG. 8

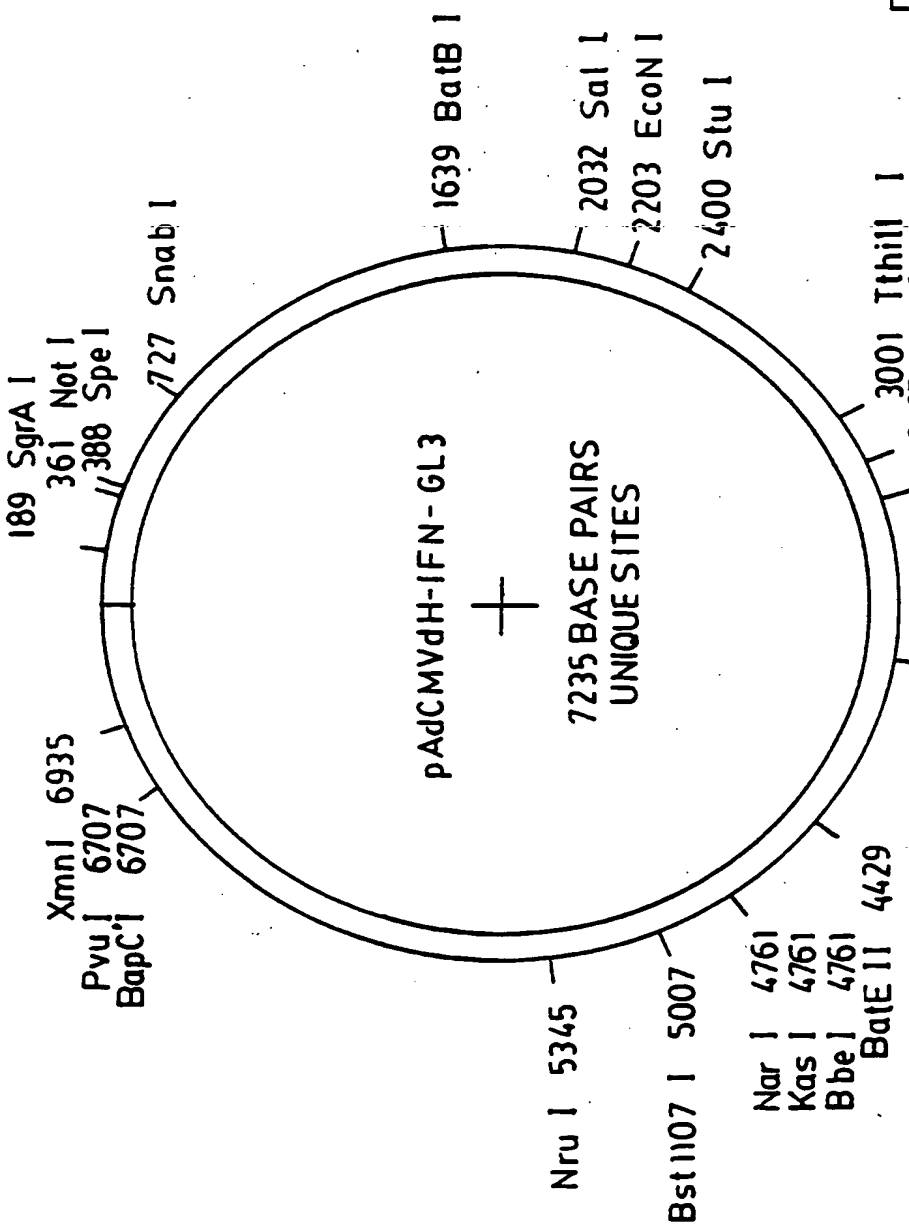

pAdCMVdH-IFN-GL3
7235 BASE PAIRS
UNIQUE SITES
189 SgrA I
361 Not I
388 Spe I
727 SnaB I
1639 BatB I
2032 Sal I
2203 EcoN I
2400 Stu I
3001 TthIII I
3167 Mun I
3304 BaaB I
3846 BatX I
4429 BatE II
4761 Nar I
4761 Kas I
4761 Bbe I
5007 Bst1107 I
5345 Nru I
6707 BapC I
6707 Pvu I
6935 Xmn I

FIG.10

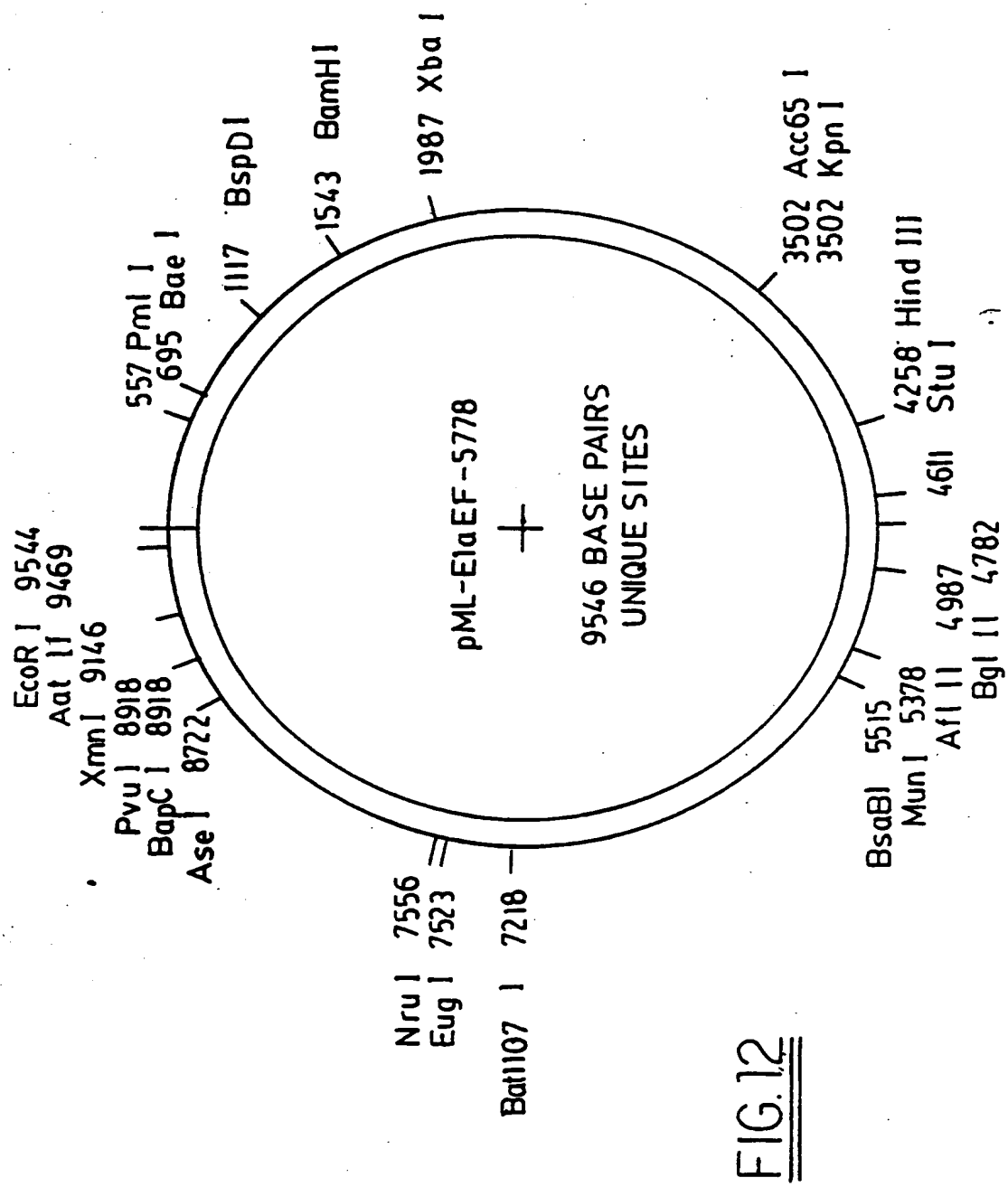

pML-E1aEF-5778
9546 BASE PAIRS
UNIQUE SITES
1987 Xba I
1543 BamH I
1117 BspD I
557 Pml I
695 Bae I
EcoR I 9544
Aat II 9469
Xmn I 9146
Pvu I 8918
BapC I 8918
Ase I 8722
Nru I 7556
Eug I 7523
Bal1107 I 7218
BsaBI 5515
Mun I 5378
Afl II 4987
Bgl II 4782
4611
4258 Stu I
4258 Hind III
3502 Acc65 I
3502 Kpn I

FIG. 12

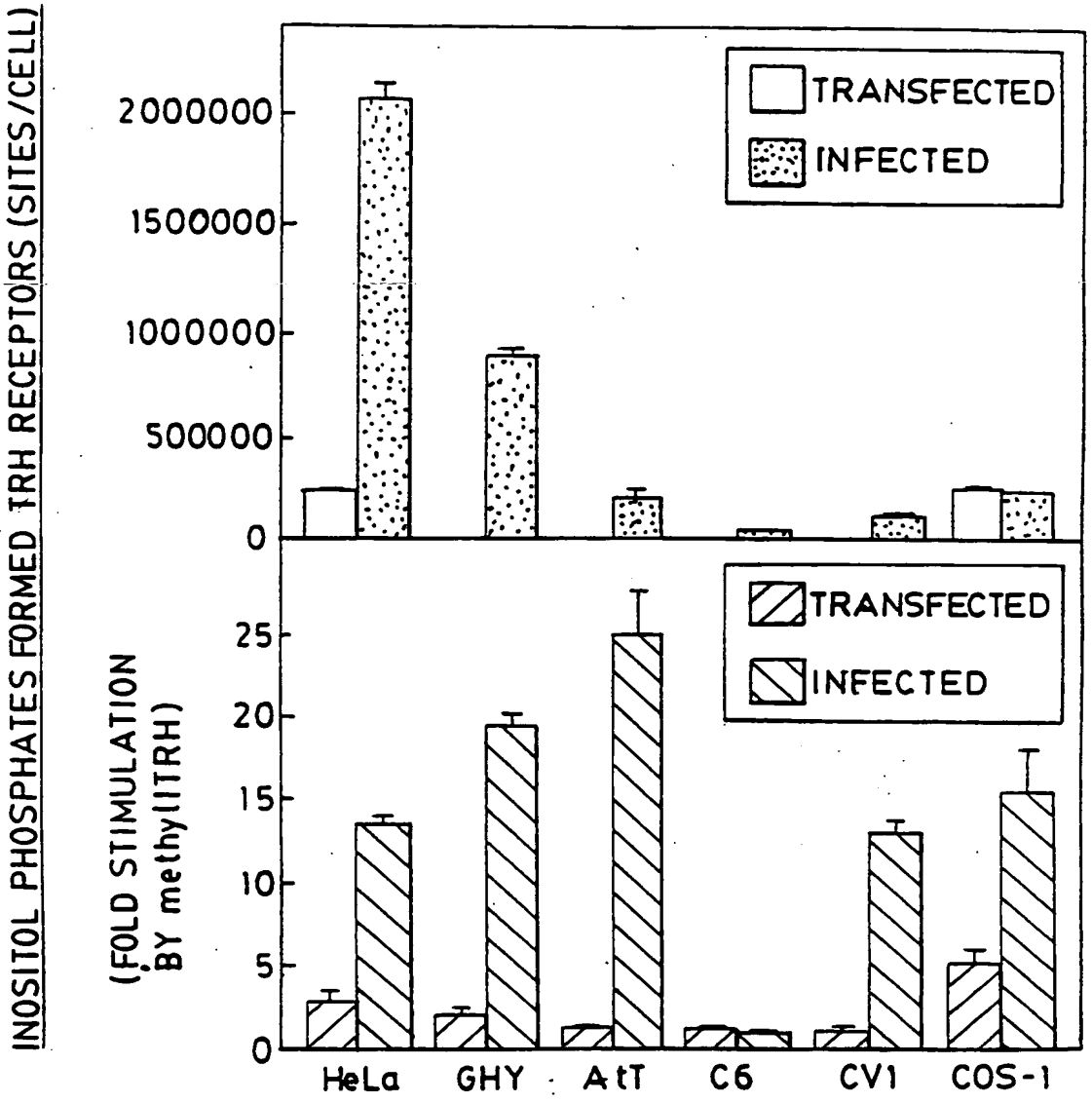

INOSITOL PHOSPHATES FORMED TRH RECEPTORS (SITES/CELL)
2000000
1500000
1000000
500000
0
TRANSFECTED
INFECTED
(FOLD STIMULATION BY methyl(1TRH)
25
20
15
10
5
0
TRANSFECTED
INFECTED
HeLa
GHY
AtT
C6
CV1
COS-1

FIG.14

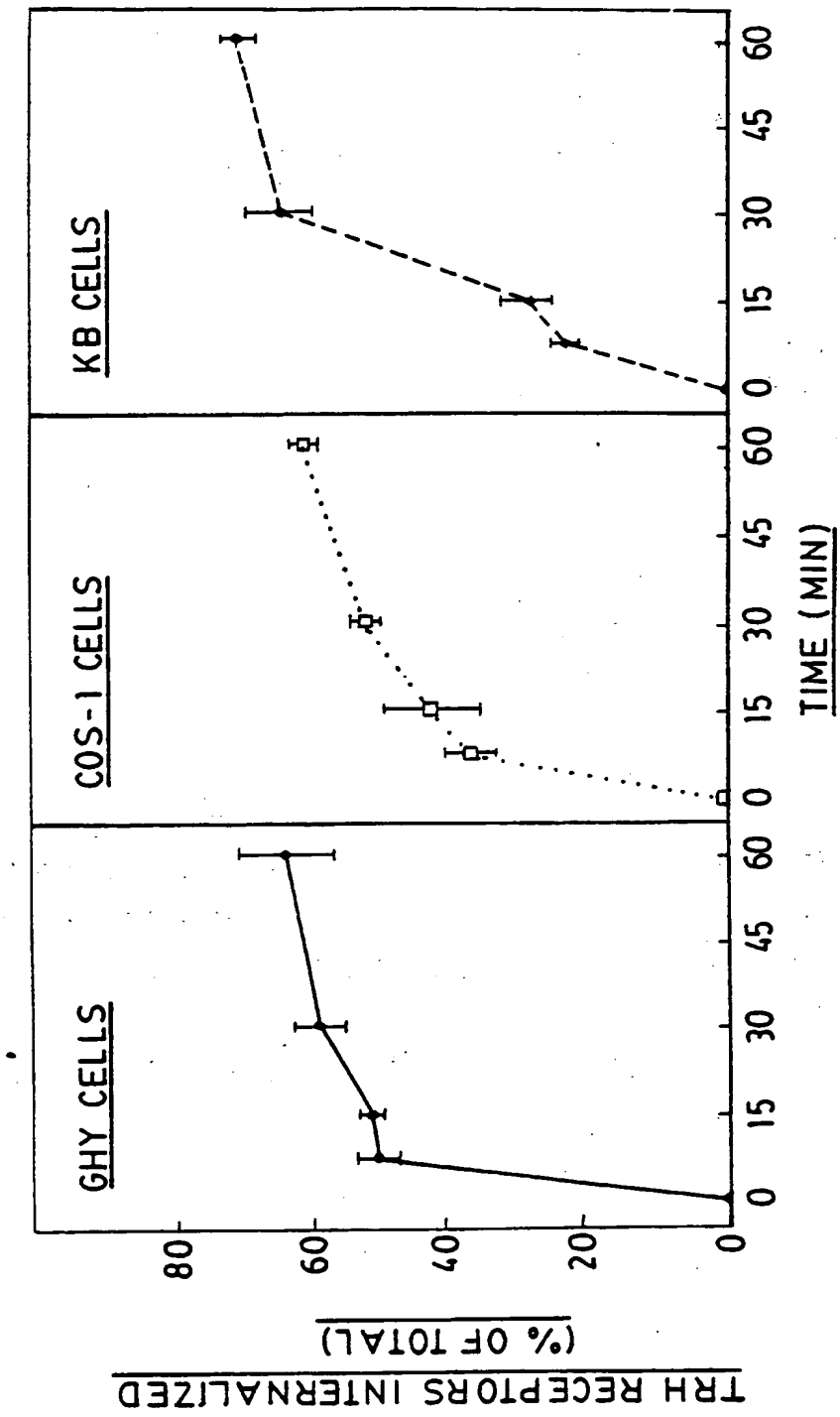

GHY CELLS
COS-1 CELLS
KB CELLS
0
15
30
45
60
0
20
40
60
80
TIME (MIN)
TRH RECEPTORS INTERNALIZED
(% OF TOTAL)

FIG.16

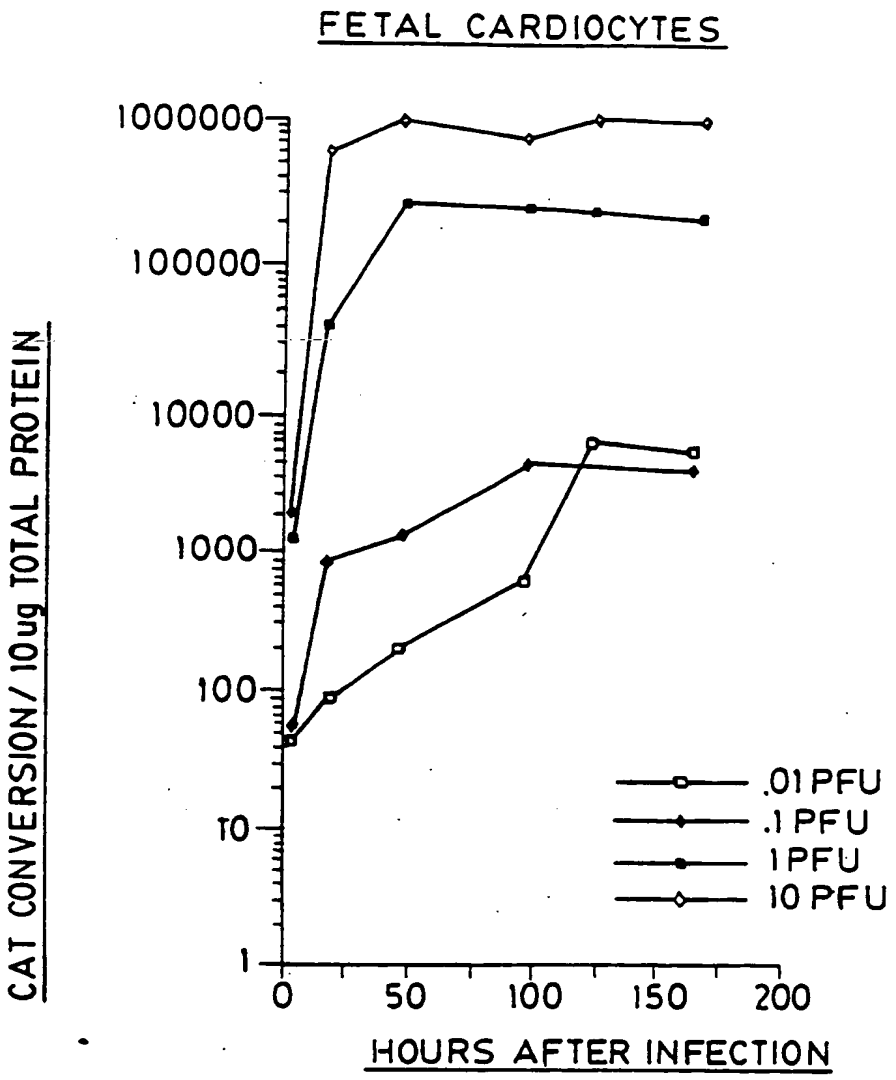

FETAL CARDIOCYTES
CAT CONVERSION/10ug TOTAL PROTEIN
1000000
100000
10000
1000
100
10
1
0
50
100
150
200
HOURS AFTER INFECTION
.01 PFU
.1 PFU
1 PFU
10 PFU

FIG. 18A

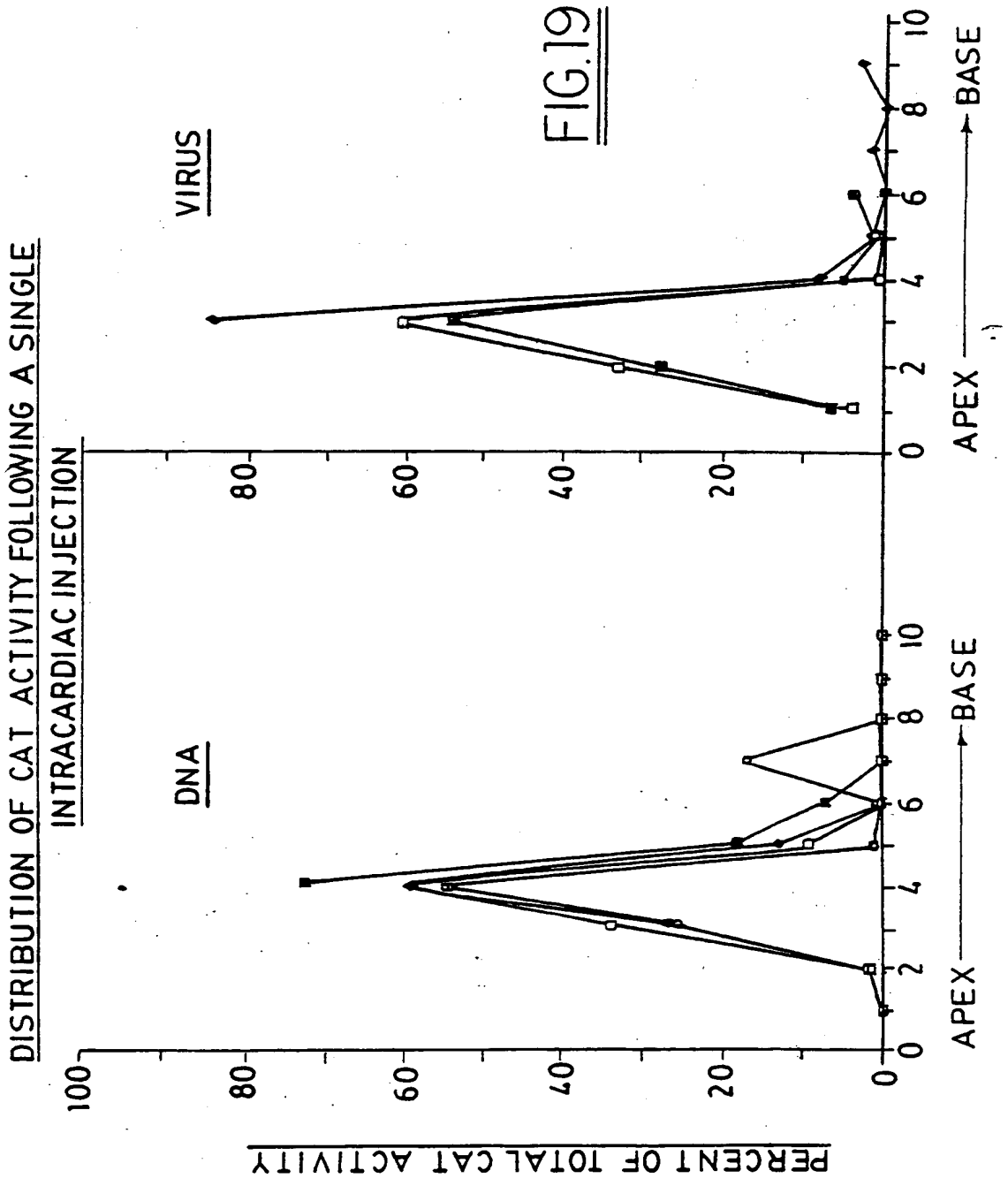
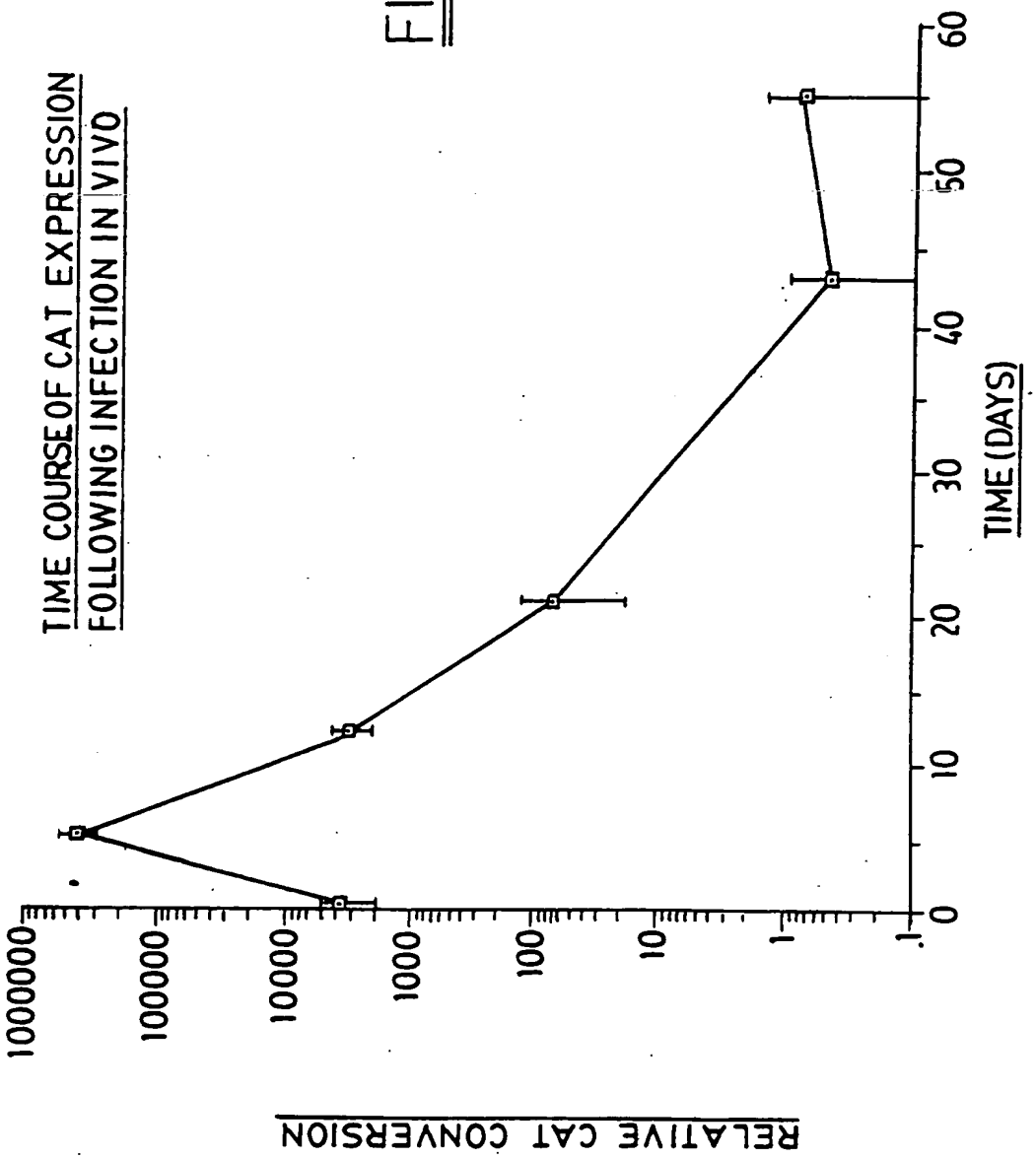

DISTRIBUTION OF CAT ACTIVITY FOLLOWING A SINGLE INTRACARDIAC INJECTION
DNA
VIRUS
PERCENT OF TOTAL CAT ACTIVITY
100
80
60
40
20
0
0
2
4
6
8
10
APEX
BASE

FIG.19

FIG. 20B


TIME COURSE OF CAT EXPRESSION
FOLLOWING INFECTION IN VIVO
RELATIVE CAT CONVERSION
1000000
100000
10000
1000
100
10
1
.1
0
10
20
30
40
50
60
TIME (DAYS)

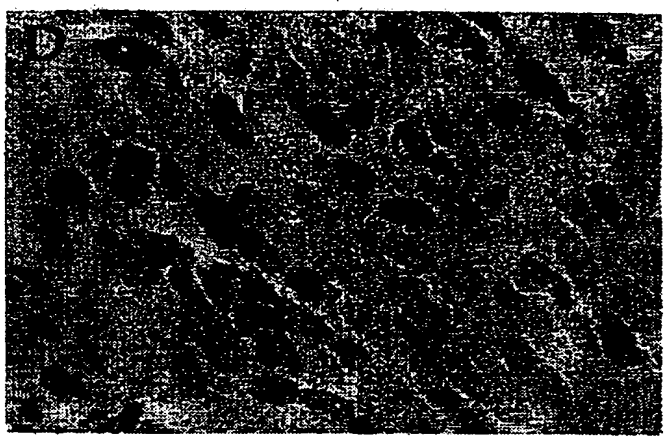


FIG. 21D

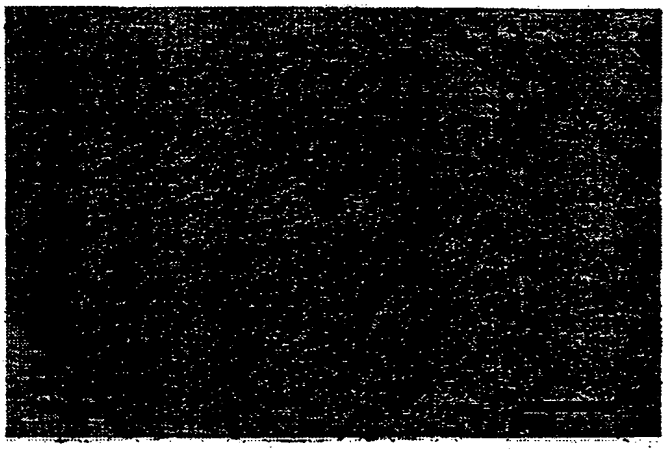


FIG. 21E

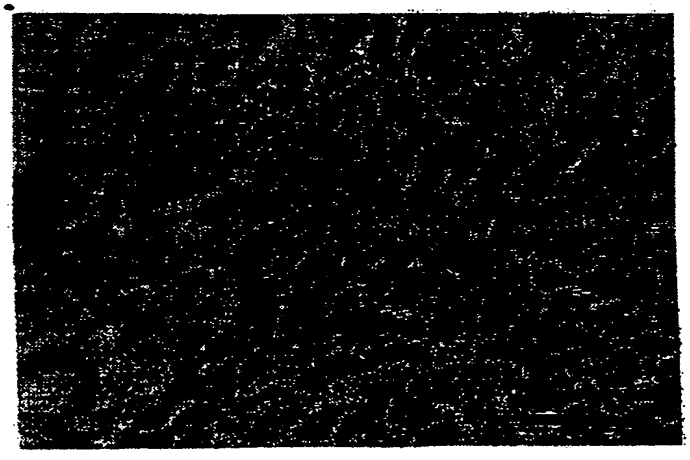


FIG. 21F

Fig. 22 (continued)

ATATTCACCT GGCCCGCGGT GATGCCTTTG AGGGTGGCCG CGTCCATCTG
GTCAGAAAAG 1140

ACAATCTTTT TGTTGTCAAA AGCGCTTGAG GTGTGGCAGG CTTGAGATCT
GGCCATACAC 1200

TTGAGTGACA ATGACATCCA CTTTGCCTTT CTCTCCACAG GTGTCCACTC
CCAGGTCCAA 1260

CTGCAGCCCC CAAGCTTGGG AATTCTCTCG GAAACGATGA AATATACAAG
TTATATCTTG 1320

GCTTTTCAGC TCTGCATCGT TTTGGGTTCT CTTGGCTGTT ACTGCCAGGA
CCCATATGTA 1380

AAAGAAGCAG AAAACCTTAA GAAATATTTT AATGCAGGTC ATTCAGATGT
AGCGGATAAT 1440

GGAACTCTTT TCTTAGGCAT TTTGAAGAAT TGGAAAGAGG AGAGTGACAG
AAAAATAATG 1500

CAGAGCCAAA TTGTCTCCTT TTACTTCAAA CTTTTTAAAA ACTTTAAAGA
TGACCAGAGC 1560

ATCCAAAAGA GTGTGGAGAC CATCAAGGAA GACATGAATG TCAAGTTTTT
CAATAGCAAC 1620

AAAAAGAAAC GAGATGACTT CGAAAAGCTG ACTAATTATT CGGTAACTGA
CTTGAATGTC 1680

CAACGCAAAG CAATACATGA ACTCATCCAA GTGATGGCTG AACTGTCGCC
AGCAGCTAAA 1740

ACAGGGAAGC GAAAAAGGAG TCAGATGCTG TTTCAAGGTC GAAGAGCATC
CCAGTAATGG 1800

TTGTCCTGCG GATCCCTGCC AGTGGCGCAT AGCGATGCGC GGCAGAACCC
CTTTGATTTT 1860

TAAACGGCGC AGACGGCAAG GGTGGGGGGT AAATAATCAC CCGAGAGTGT
ACAAATAAAA 1920

ACATTTGCCT TTATTGAAAG TGTCTCCTAG TACATTATTT TTACATGTTT
TTCAAGTGAC 1980

AAAAAGAAGT GGCGCTCCTA ATCTGCGCAC TGTGGCTGCG GGAGCTCTAG
AGTCGACGGT 2040

ATCGCCCGAC ATCACCTGTG TCTATGGCCA CTGCCTTGGC TCACAAGTAC
CACTAAACCC 2100

CCTTTCCTGC TCTTGCCTGT GAACAATGGT TAATTGTTCC CAAGAGAGCA
TCTGTCAGTT 2160

Fig. 22 (continued)

GCGGGATTGT GACTGACTTT GCTTTCCTGA GCCCGCTTGC AAGCAGTGCA
GCTTCCCGTT 3300

CATCCGCCCG CGATGACAAG TTGACGGCTC TTTTGGCACA ATTGGATTCT
TTGACCCGGG 3360

AACTTAATGT CGTTTCTCAG CAGCTGTTGG ATCTGCGCCA GCAGGTTTCT
GCCCTGAAGG 3420

CTTCCTCCCC TCCCAATGCG GTTTAAAACA TAAATAAAAA ACCAGACTCT
GTTTGGATTT 3480

GGATCAAGCA AGTGTCTTGC TGTCTTTATT TAGGGGTTTT GCGCGCGCGG
TAGGCCCGGG 3540

ACCAGCGGTC TCGGTCGTTG AGGGTCCTGT GTATTTTTTC CAGGACGTGG
TAAAGGTGAC 3600

TCTGGATGTT CAGATACATG GGCATAAGCC CGTCTCTGGG GTGGAGGTAG
CACCACTGCA 3660

GAGCTTCATG CTGCGGGGTG GTGTTGTAGA TGATCCAGTC GTAGCAGGAG
CGCTGGGCGT 3720

GGTGCCTAAA AATGTCTTTC AGTAGCAAGC TGATTGCCAG GGGCAGGCCC
TTGGTGTAAG 3780

TGTTTACAAA GCGGTTAAGC TGGGATGGGT GCATACGTGG GGATATGAGA
TGCATCTTGG 3840

ACTGTATTTT TAGGTTGGCT ATGTTCCCAG CCATATCCCT CCGGGGATTC
ATGTTGTGCA 3900

GAACCACCAG CACAGTGTAT CCGGTGCACT TGGGAAATTT GTCATGTAGC
TTAGAAGGAA 3960

ATGCGTGGAA GAACTTGGAG ACGCCCTTGT GACCTCCAAG ATTTTCCATG
CATTCGTCCA 4020

TAATGATGGC AATGGGCCCA CGGGCGGCGG CCTGGGCGAA GATATTTCTG
GGATCACTAA 4080

CGTCATAGTT GTGTTCCAGG ATGAGATCGT CATAGGCCAT TTTTACAAAG
CGCGGGCGGA 4140

GGGTGCCAGA CTGCGGTATA ATGGTTCCAT CCGGCCCAGG GGCGTAGTTA
CCCTCACAGA 4200

TTTGCATTTC CCACGCTTTG AGTTCAGATG GGGGGATCAT GTCTACCTGC
GGGGCGATGA 4260

AGAAAACGGT TTCCGGGGTA GGGGAGATCA GCTGGGAAGA AAGCAGGTTC
CTGAGCAGCT 4320

Fig. 22 (continued)

CTTGCACGCC CTCGCTCAAG CCTTCGTCAC TGGTCCCGCC ACCAAACGTT
TCGGCGAGAA 5460

GCAGGCCATT ATCGCCGGCA TGGCGGCCGA CGCGCTGGGC TACGTCTTGC
TGGCGTTCGC 5520

GACGCGAGGC TGGATGGCCT TCCCCATTAT GATTCTTCTC GCTTCCGGCG
GCATCGGGAT 5580

GCCCGCGTTG CAGGCCATGC TGTCCAGGCA GGTAGATGAC GACCATCAGG
GACAGCTTCA 5640

AGGATCGCTC GCGGGTAAAA AGGCCGCGTT GCTGGCGTTT TTCCATAGGC
TCCGCCCCCC 5700

TGACGAGCAT CACAAAAATC GACGCTCAAG TCAGAGGTGG CGAAACCCGA
CAGGACTATA 5760

AAGATACCAG GCGTTTCCCC CTGGAAGCTC CCTCGTGCGC TCTCCTGTTC
CGACCCTGCC 5820

GCTTACCGGA TACCTGTCCG CCTTTCTCCC TTCGGGAAGC GTGGCGCTTT
CTCAATGCTC 5880

ACGCTGTAGG TATCTCAGTT CGGTGTAGGT CGTTCGCTCC AAGCTGGGCT
GTGTGCACGA 5940

ACCCCCCGTT CAGCCCGACC GCTGCGCCTT ATCCGGTAAC TATCGTCTTG
AGTCCAACCC 6000

GGTAAGACAC GACTTATCGC CACTGGCAGC AGCCACTGGT AACAGGATTA
GCAGAGCGAG 6060

GTATGTAGGC GGTGCTACAG AGTTCTTGAA GTGGTGGCCT AACTACGGCT
ACACTAGAAG 6120

GACAGTATTT GGTATCTGCG CTCTGCTGAA GCCAGTTACC TTCGGAAAAA
GAGTTGGTAG 6180

CTCTTGATCC GGCAAACAAA CCACCGCTGG TAGCGGTGGT TTTTTTGTTT
GCAAGCAGCA 6240

GATTACGCGC AGAAAAAAAG GATCTCAAGA AGATCCTTTG ATCTTTTCTA
CGGGGTCTGA 6300

CGCTCAGTGG AACGAAAACT CACGTTAAGG GATTTTGGTC ATGAGATTAT
CAAAAAGGAT 6360

CTTCACCTAG ATCCTTTTAA ATTAAAAATG AAGTTTTAAA TCAATCTAAA
GTATATATGA 6420

GTAAACTTGG TCTGACAGTT ACCAATGCTT AATCAGTGAG GCACCTATCT
CAGCGATCTG 6480

Fig. 23

TTCCATCATC AATAATATAC CTTATTTTGG ATTGAAGCCA ATATGATAAT
GAGGGGGTGG 60

AGTTTGTGAC GTGGCGCGGG GCGTGGGAAC GGGGCGGGTG ACGTAGTAGT
GTGGCGGAAG 120

TGTGATGTTG CAAGTGTGGC GGAACACATG TAAGCGACGG ATGTGGCAAA
AGTGACGTTT 180

TTGGTGTGCG CCGGTGTACA CAGGAAGTGA CAATTTTCGC GCGGTTTTAG
GCGGATGTTG 240

TAGTAAATTT GGGCGTAACC GAGTAAGATT TGGCCATTTT CGCGGGAAAA
CTGAATAAGA 300

GGAAGTGAAA TCTGAATAAT TTTGTGTTAC TCATAGCGCG TAATATTTGT
CTAGGGCCTT 360

GCGGCCGCAA GTTGACATTG ATTATTGACT AGTTATTAAT AGTAATCAAT
TACGGGGTCA 420

TTAGTTCATA GCCCATATAT GGAGTTCCGA GTTACATAAC TTACGGTAAA
TGGCCCGCCT 480

GGCTGACCGC CCAACGACCC CCGCCCATTG ACGTCAATAA TGACGTATGT
TCCCATAGTA 540

ACGCGAATAG GGACTTTCCA TTGACGTCAA TGGGTGGAGT ATTTACGGTA
AACTGCCCAC 600

TTGGCAGTAC ATCAAGTGTA TCATATGCCA AGTACGCCCC CTATTGACGT
CAATGACGGT 660

AAATGGCCCG CCTGGCATTA TGCCCAGTAC ATGACCTTAT GGGACTTTCC
TACTTGGCAG 720

TACATCTACG TATTAGTCAT CGCTATTACC ATGGTGATGC GGTTTTGGCA
GTACATCAAT 780

GGGCGTGGAT AGCGGTTTGA CTCACGGGGA TTTCCAAGTC TCCACCCCAT
TGACGTCAAT 840

GGGAGTTTGT TTTGGCACCA AAATCAACGG GACTTTCCAA AATGTCGTAA
CAACTCCGCC 900

CCATTGACGC AAAGGGTCGG TAGGCGTGTA CGGTGGGAGG TCTATATAAG
CAGAGCTCGC 960

CCGGGGATCC TCTAGAATTC GCTGTCTGCG AGGGCCAGCT GTTGGGGTGA
GTACTCCCTC 1020

Fig. 23 (continued)

CGATGAGACC CGCACCAGGT GCAGACCCTG CGAGTGTGGC GGTAAACATA
TTAGGAACCA 2100

GCCTGTGATG CTGGATGTGA CCGAGGAGCT GAGGCCCGAT CACTTGGTGC
TGGCCTGCAC 2160

CCGCGCTGAG TTTGGCTCTA GCTATGAAGA TACAGATTGA GGTACTGAAA
TGTGTGGGCG 2220

TGGCTTAAGG GTGGGAAAGA ATATATAAGG TGGGGGTCTT ATGTAGTTTT
GTATCTGTTT 2280

TGCAGCAGCC GCCGCCGCCA TGAGCACCAA CTCGTTTGAT GGAAGCATTG
TGAGCTCATA 2340

TTTGACAACG CGCATGCCCC CATGGGCCGG GGTGCGTCAG AATGTGATGG
GCTCCAGCAT 2400

TGATGGTCGC CCCGTCCTGC CCGCAAACTC TACTACCTTG ACCTACGAGA
CCGTGTCTGG 2460

AACGCCGTTG GAGACTGCAG CCTCCGCCGC CGCTTCAGCC GCTGCAGCCA
CCGCCCGCGG 2520

GATTGTGACT GACTTTGCTT TCCTGAGCCC GCTTGCAAGC AGTGCAGCTT
CCCGTTCATC 2580

CGCCCGCGAT GACAAGTTGA CGGCTCTTTT GGCACAATTG GATTCTTTGA
CCCGGGAACT 2640

TAATGTCGTT TCTCAGCAGC TGTTGGATCT GCGCCAGCAG GTTTCTGCCC
TGAAGGCTTC 2700

CTCCCCTCCC AATGCGGTTT AAAACATAAA TAAAAAACCA GACTCTGTTT
GGATTTGGAT 2760

CAAGCAAGTG TCTTGCTGTC TTTATTTAGG GGTTTTGCGC GCGCGGTAGG
CCCGGGACCA 2820

GCGGTCTCGG TCGTTGAGGG TCCTGTGTAT TTTTTCCAGG ACGTGGTAAA
GGTGACTCTG 2880

GATGTTCAGA TACATGGGCA TAAGCCCGTC TCTGGGGTGG AGGTAGCACC
ACTGCAGAGC 2940

TTCATGCTGC GGGGTGGTGT TGTAGATGAT CCAGTCGTAG CAGGAGCGCT
GGGCGTGGTG 3000

CCTAAAAATG TCTTTCAGTA GCAAGCTGAT TGCCAGGGGC AGGCCCTTGG
TGTAAGTGTT 3060

Fig. 23 (continued)

CTGGTCCTGC TGGTGCTGAA GCGCTGCCGG TCTTCGCCCT GCGCGTCGGC
CAGGTAGCAT 4140

TTGACCATGG TGTCATAGTC CAGCCCCTCC GCGGCGTGGC CCTTGGCGCG
CAGCTTGCCC 4200

TTGGAGGAGG CGCCGCACGA GGGGCAGTGC AGACTTTTGA GGGCGTAGAG
CTTGGGCGCG 4260

AGAAATACCG ATTCCGGGGA GTAGGCATCC GCGCCGCAGG CCCCGCAGAC
GGTCTCGCAT 4320

TCCACGAGCC AGGTGAGCTC TGGCCGTTCG GGGTCAAAAA CCAGGTTTCC
CCCATGCTTT 4380

TTGATGCGTT TCTTACCTCT GGTTTCCATG AGCCGGTGTC CACGCTCGGT
GACGAAAAGG 4440

CTGTCCGTGT CCCCGTATAC AGACTTGAGA GGTCGAGCGA TGCCCTTGAG
AGCCTTCAAC 4500

CCAGTCAGCT CCTTCCGGTG GGCGCGGGGC ATGACTATCG TCGCCGCACT
TATGACTGTC 4560

TTCTTTATCA TGCAACTCGT AGGACAGGTG CCGGCAGCGC TCTGGGTCAT
TTTCGGCGAG 4620

GACCGCTTTC GCTGGAGCGC GACGATGATC GGCCTGTCGC TTGCGGTATT
CGGAATCTTG 4680

CACGCCCTCG CTCAAGCCTT CGTCACTGGT CCCGCCACCA AACGTTTCGG
CGAGAAGCAG 4740

GCCATTATCG CCGGCATGGC GGCCGACGCG CTGGGCTACG TCTTGCTGGC
GTTCGCGACG 4800

CGAGGCTGGA TGGCCTTCCC CATTATGATT CTTCTCGCTT CCGGCGGCAT
CGGGATGCCC 4860

GCGTTGCAGG CCATGCTGTC CAGGCAGGTA GATGACGACC ATCAGGGACA
GCTTCAAGGA 4920

TCGCTCGCGG GTAAAAAGGC CGCGTTGCTG GCGTTTTTCC ATAGGCTCCG
CCCCCCTGAC 4980

GAGCATCACA AAAATCGACG CTCAAGTCAG AGGTGGCGAA ACCCGACAGG
ACTATAAAGA 5040

TACCAGGCGT TTCCCCCTGG AAGCTCCCTC GTGCGCTCTC CTGTTCCGAG
CCTGCCGCTT 5100

Fig. 23 (continued)

TTGTGCAAAA AAGCGGTTAG CTCCTTCGGT CCTCCGATCG TTGTCAGAAG
TAAGTTGGCC 6180

GCAGTGTTAT CACTCATGGT TATGGCAGCA CTGCATAATT CTCTTACTGT
CATGCCATCC 6240

GTAAGATGCT TTTCTGTGAC TGGTGAGTAC TCAACCAAGT CATTCTGAGA
ATAGTGTATG 6300

CGGCGACCGA GTTGCTCTTG CCCGGCGTCA ACACGGGATA ATACCGCGCC
ACATAGCAGA 6360

ACTTTAAAAG TGCTCATCAT TGGAAAACGT TCTTCGGGGC GAAAACTCTC
AAGGATCTTA 6420

CCGCTGTTGA GATCCAGTTC GATGTAACCC ACTCGTGCAC CCAACTGATC
TTCAGCATCT 6480

TTTACTTTCA CCAGCGTTTC TGGGTGAGCA AAAACAGGAA GGCAAAATGC
CGCAAAAAAG 6540

GGAATAAGGG CGACACGGAA ATGTTGAATA CTCATACTCT TCCTTTTTCA
ATATTATTGA 6600

AGCATTTATC AGGGTTATTG TCTCATGAGC GGATACATAT TTGAATGTAT
TTAGAAAAAT 6660

AAACAAATAG GGGTTCCGCG CACATTTCCC CGAAAAGTGC CACCTGACGT
CTAAGAAACC 6720

ATTATTATCA TGACATTAAC CTATTAAAAT AGGCGTATCA CGAGGCCCTT
TCGTCTTCAA 6780

GAA

6783

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/14502

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. Clin. Invest., Volume 92, issued July 1993, L. A. Kirshenbaum et al., "Highly Efficient Gene Transfer into Adult Ventricular Myocytes by Recombinant Adenovirus," pages 381-387, see entire article.	1-16
Y	Proc. Natl. Acad. Sci. USA, Volume 89, issued April 1992, B. Quantin et al., "Adenovirus as an expression vector in muscle cells in vivo," pages 2581-2584, see entire article.	1-16
P,X	Proc. Natl. Acad. Sci. USA, Volume 90, issued 15 December 1993, A. Kass-Eisler et al., "Quantitative determination of adenovirus-mediated gene delivery to rat cardiac myocytes in vitro and in vivo," pages 11498-11502, see entire article.	1-16